PHYLOGENY OF MYRTEAE (MYRTACEAE) WITH AN EMPHASIS ON THE CHILEAN SPECIES: INSIGHTS INTO CHARACTER EVOLUTION AND HISTORICAL BIOGEOGRAPHY

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Abstract

The predominantly southern hemisphere tribe Myrteae (Myrtaceae) is represented in Chile by 25 species in nine genera. Chilean Myrteae inhabit a broad range of environments including humid temperate forests, riparian habitats, swamps and coastal xerophytic shrublands. Seventeen species are endemic to Chile, eight occur in Chile and Argentina and one species is distributed from Chile-Argentina to Venezuela along the Andes. Phylogenetic studies have investigated the relationships in Myrteae, but the systematic position of some Chilean (e.g., *Ugni candollei*, *Myrcianthes coquimbensis*) and affiliated species remains unclear and major nodes have low statistical support. The use of morphological characters to investigate phylogenetic relationships has not been undertaken in the tribe. The phylogenetic placement, historical biogeography and character evolution of all the Chilean Myrteae were investigated in this study. The sampling strategy ensured that all major nodes in Myrteae were represented (total 101 species). Outgroups (10 species) included representatives from seven tribes of Myrtaceae. Phylogenetic analyses of DNA sequences from four loci as well as 79 morphological characters were performed using parsimony, maximum likelihood and Bayesian inference. The inclusion of morphological characters improved resolution and statistical support for some clades. As determined by other studies, the tribe Myrteae is monophyletic with strong statistical support. The Chilean Myrteae do not form a monophyletic group, instead they are distributed in six lineages and in most cases form the sister taxon of to the rest of the clade. Some results show that *Ugni* is not monophyletic and divided into two clades (*U. candollei* + *U. selkirkii* and *U. molinae* + *U. myricoides*) with unclear phylogenetic position. An interesting result from this study is that the Chilean endemic *Myrcianthes coquimbensis* is sister to all other species of *Myrcianthes* with strong statistical support. This investigation includes the first comprehensive study of the foliar and floral anatomy of Chilean Myrtaceae. The majority of species have a typical mesophytic leaf, with a few exceptions (e.g., *Myrceugenia rufa*, *Myrcianthes coquimbensis*), which possess xerophytic and/or sclerophyllous characters such as the presence of a hypodermis, sunken stomata and a dense layer of foliar hairs. Morphological synapomorphies include dibrachiate hairs in the genus *Myrceugenia*, independent origin of paracytic stomata in *Luma* and *Psidium* and an arc-shaped leaf vascular system in *Myrceugenia*. Several morphological characters (e.g., hypodermis, multiple epidermis, glandular hairs, circular midrib, absence of secretory cavities in anthers) are homoplasious and have evolved a number of times during the evolutionary history of Myrteae. Biogeographic analyses support a Gondwanan origin of Myrteae with *Myrtus* occurring in the Mediterranean probably as result of a dispersal event. The inclusion of additional genera restricted to Brazil, Argentina and Australasia and the use of additional variable molecular loci are required to gain a more complete understanding of the phylogenetic relationships and biogeography of the tribe Myrteae.

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Definition of terms

Anatomy (plants): The study of the internal structure and cellular organization of plants. It involves the sectioning of organs and structures[2].

Character: A characteristic possessed by a taxon (e.g., morphological, molecular) [1].

Clade: A taxon (group of organisms) consisting of a common ancestor and all its descendants (i.e., a monophyletic group) [1].

Histochemistry: The study of the chemical compounds that form part and/or are produced by cells and tissues[2].

Homoplasy (Homoplastic character state): Character state observed in two or more species because of parallel or convergent evolution[1].

Monophyletic group: A group consisting of a common ancestor and all its descendants[1].

Morphology: External characteristics of an organism or group of organisms[2].

Paraphyletic group: A group that contains the most recent common ancestor but not all of its descendants[1].

Phylogenetics: Techniques used to study the evolutionary relationships among organisms[3].

Phylogeny: The hypothetical evolutionary history of a group of organisms, commonly represented as a phylogenetic tree[1].

Polyphyletic group: A group that does not include the most recent common ancestor of all descendants of the group[1].

Synapomorphy: Shared derived characters that define monophyletic groups[1].

Systematics: The field of science that studies the nomenclature and classification of organisms in an evolutionary context

Taxonomy: The field of science that studies the description, identification, nomenclature and classification of organisms[3].

Sources: Wiens (2000)**[1]**, Evert (2006)**[2]** and Scharaschkin (2013)**[3]**.

Abbreviations

BI: Bayesian Inference

BS: Bootstrap support

Bp: Base pairs (DNA)

CTAB: Cetyltrimethyl ammonium bromide

LDD: Long distance dispersal

LM: Light microscopy

Mya: Million years ago

SEM: Scanning electron microscopy

MP: Maximum Parsimony

PP: Posterior Probability (Bayesian probability)

Syn: synonym (taxonomic synonym)

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Statement of original authorship

The work contained in this thesis has not been previously submitted to meet requirements for an award, degree or diploma at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

Signed:

Date: 01 February 2017

CHAPTER 1: General introduction

This chapter provides a general overview of the research problem and an explanation of how the thesis is structured and organized. The chapter is divided into three sections, namely (1) description of the research problem investigated, (2) aims of the study and (3) outline of the thesis.

1.1 Description of the research problem investigated

Myrtaceae Juss. is an angiosperm family of trees and shrubs that belongs to the order Myrtales (APG IV, 2016). The family is divided into two subfamilies (Myrtoideae and Psiloxyloideae), 17 tribes and 142 genera and includes more than 5000 species (Wilson et al., 2005; Govaerts et al., 2008). Representatives of Myrtaceae include myrtles, eucalypts, lilly-pillies, cloves and guavas (Wilson, 2011). The family is mainly distributed in the southern hemisphere, and is especially diverse in South and Central America and Australia (Ladiges et al., 2003). The species occur in a wide range of habitats, including seasonally dry areas, coastal sands, rainforests, tropical moist forests, savannahs and mangroves (Biffin et al., 2010; Wilson, 2011). The tribe Myrteae is the largest of the 17 tribes in Myrtaceae. All South American Myrtaceae belong to Myrteae, other than *Metrosideros stipularis* (Hook. and Arn.) Hook.f. (syn. *Tepualia stipularis*, WCSP, 2016 in the tribe Metrosidereae, (Wilson, 2011; Pillon et al., 2015).

In Chile, the tribe Myrteae is represented by 25 species (plus one variety) in nine genera and occur from the centre-north to the southern tip of the mainland region and in the Juan Fernandez Archipelago (Landrum, 1988a; Murillo and Ruiz, 2011). Most of the Chilean Myrteae genera are monotypic, bitypic or have a limited number of species, which has been related to the early isolation of the Chilean flora and to the particular geographical barriers present in Chile (Villagran and Hinojosa, 1997; Moreira-Muñoz, 2011). All the species are either endemic to Chile or endemic to the humid temperate forests of Chile and Argentina, with the exception of *Myrteola nummularia*, which occurs in Chile, Argentina and the Andean regions of Venezuela, Colombia, Ecuador, Peru and Bolivia (Landrum, 1988a).

Phylogenetic studies in the tribe Myrteae have aimed to resolve relationships at tribal or suprageneric level (Lucas et al., 2005, 2007; Biffin et al., 2010) or on a particular genus (for example, Murillo et al., 2012, 2013, 2016). Some Chilean Myrteae have been included as

representatives of some genera in such studies. Such studies have not been able to determine the phylogenetic position of all Chilean Myrteae, due to incomplete sampling or insufficiently variable data. Specific issues that need attention include the addition of Chilean endemic species (e.g., *Myrcianthes coquimbensis* and *Ugni candollei*), which may contribute to elucidate the phylogenetic relationships of these two genera. The monophyly of the small genera *Ugni* (four species, three in Chile and one distributed in Central America and northern South America) and *Blepharocalyx* (three species, one in Chile and two in South-Central America) has never been tested. Some Chilean genera (*Amomyrtus*, *Luma*, *Myrceugenia*) have been confirmed monophyletic (Lucas et al., 2007). However, an improved taxonomic sampling has been recommended in order to elucidate relationships between genera such as *Blepharocalyx*, *Luma*, *Ugni* and *Myrcianthes* (Lucas et al., 2007, Biffin et al., 2010; Murillo et al., 2013). The phylogenetic position of the two Chilean varieties of *Myrceugenia ovata* (*var. ovata* and *var. nanophylla*) needs more attention, since these two varieties appear sister in some studies (Murillo et al., 2016) and not in others (Landrum, 1981a; Murillo et al., 2013).

Chilean species of Myrteae have played a key role in previous phylogenetic analyses of the tribe, which highlights the importance of these species in the biogeographic context of South America. The significance of the Chilean Myrteae is based on phylogenetic studies that have shown Chilean representatives as sister group to eastern South American or Australasian genera (Lucas et al., 2007; Murillo et al., 2013). The current geographic distribution of Myrteae has been studied and explained by a combination of vicariance and long distance dispersal events (Sytsma et al., 2004; Biffin et al., 2010; Murillo et al., 2016). Examples of vicariance include the presence of the tribe in South America, Africa and Australasia due to the break-up of Gondwana and the existence of the Antarctic land bridge during the Eocene (Johnson and Briggs, 1984). Dispersal events have been considered important to explain the presence of some Myrteae in Africa, New Zealand and the Mediterranean (Sytsma et al., 2004; Lucas et al., 2007; Murillo et al., 2016). *Myrtus* (Mediterranean) is the only Myrteae genus in the northern hemisphere and has been indicated as sister group to all the tribe Myrteae (Sytsma et al., 2004; Lucas et al., 2007; Biffin et al., 2010; Murillo et al., 2013, 2016). It is unclear whether the presence of *Myrtus* in Europe is result of a single dispersal event from Gondwana or the sole remaining of a past widespread Laurasian clade sister to the Southern Myrteae (Thornhill et al., 2015). The systematic position of *Myrtus* needs further investigation, since most analyses have shown only weak to moderate support for this

relationship. Phylogenetic investigations in Myrteae have been purely based on DNA sequences, while other sources of data have not been explored under a phylogenetic perspective.

The use of morphology (e.g., gross morphology, micromorphology, anatomy) in phylogenetic analyses has shown to be helpful in resolving relationships, particularly combined with molecular data (Baum, 1989; Lens et al., 2007; Doyle and Le Thomas, 2012). Inclusion of morphological characters can contribute to increase resolution and statistical support (Scharaschkin and Doyle, 2006; Prevosti and Chemisquy, 2010). Along with gross morphology and anatomical characters, some histochemical characters are regarded as potentially informative for evolutionary studies in a number of families, including Myrtaceae (Schmid, 1980; Judd et al., 2008; Wink, 2011). The interpretation of histochemical characters strongly depends on the protocol used and its applicability to a certain family/group of plants (Johansen, 1940; Ciccarelli et al., 2008). The significance of morphological characters for phylogenetic reconstructions in the tribe Myrteae is uncertain, since their inclusion has yet to be comprehensively conducted at tribal level. Micromorphological and anatomical information is scarce in Myrteae and particularly in Chilean species, which prevents the construction of a robust morphological data set for phylogenetic studies.

Combination of DNA sequences and a comprehensive data set of morphological characters (e.g., gross morphology, micromorphology, anatomy, histochemistry) may provide a robust and well-supported phylogeny of Myrteae. The use of morphological evidence along with molecular sequences might help to elucidate phylogenetic problems in Chilean Myrtaceae (i.e., the systematic position of *Myrcianthes coquimbensis*, *Ugni candollei*, *Myrceugenia ovata*, etc.). In order to reliably describe histochemical characters and facilitate the observation of some anatomical characters in Myrtaceae, an optimized histochemical protocol is needed. The construction of a robust morphological matrix that includes all the species of Chilean Myrteae needs a comprehensive study on the leaf/flower micromorphology and anatomy, which is unknown for these taxa. An understanding of the phylogenetic relationships amongst all species of Chilean Myrteae will form the basis for a discussion on character evolution and biogeography of these species in the context of the tribe Myrteae.

1.2 Aims of the study

The overall aim of the study is to determine the phylogenetic position of all the Chilean Myrtaceae and study the biogeography and character evolution in the tribe Myrteae. The specific aims of the study are to:

(1) Develop an optimized histochemical protocol to reliably describe anatomical characters and secondary compounds in different genera of Myrtaceae;
(2) Describe and compare the leaf and flower micromorphology and anatomy of all the Chilean species of Myrtaceae using scanning electron and light microscopy;
(3) Reconstruct the phylogenetic relationships in the tribe Myrteae using molecular and morphological data including a comprehensive sampling of Chilean species;
(4) Reconstruct the ancestral states of morphological characters and identify unambiguous morphological synapomorphies and re-evaluate previous historical biogeographic scenarios for Myrteae.

1.3 Thesis outline

This thesis contains eight chapters; two of which have been published and two are in advanced stage of preparation for submission to scientific journals. The following is a summary of each chapter and an explanation of how the chapters connect to each other in the thesis context.

Chapter 1 (this chapter) provides a brief description of the research problem investigated, the aims of the study and the thesis outline.

Chapter 2 is a comprehensive literature review on the taxonomy, phylogeny, morphology and biogeography of the tribe Myrteae. This chapter highlights the knowledge gaps identified at the start of the PhD process regarding the Chilean Myrteae and serves as a link between the aims listed in Chapter 1 and all subsequent chapters. The introduction section of subsequent chapters will contain a repetition of information contained in the literature review to some extent.

Chapter 3 is a methodological investigation that provides an optimized histochemical protocol to describe secondary compounds and other anatomical characters in Myrtaceae. This protocol facilitates the examination of histochemical and some anatomical characters described in Chapter 4 and Chapter 5. This chapter has been published in a scientific journal.

The published abstract and authorship details have been included Appendix 5. This chapter addresses the first specific aim of the thesis.

Chapter 4 is a comprehensive anatomical and micromorphological investigation of the leaves of all the species of Chilean Myrtaceae. In this investigation, anatomical and micromorphological characters are examined using standard LM and SEM methods and the staining protocol developed in Chapter 3. Characters described in this chapter will be included in subsequent phylogenetic analyses (Chapter 6 and 7). This chapter has been published in a scientific journal. The published abstract and authorship details have been included Appendix 5.

Chapter 5 is a study on the flower anatomy and micromorphology of some species of Chilean Myrtaceae. Characters described in this chapter will be included in subsequent phylogenetic analyses (Chapter 6 and 7). This chapter has been submitted to a scientific journal for publication. Altogether, Chapter 4 and Chapter 5 fulfil the second specific aim of this thesis. A number of relevant papers on the leaf, flower and fruit anatomy of some species have been published during the course of the PhD. The published abstracts and authorship details have been included Appendix 5.

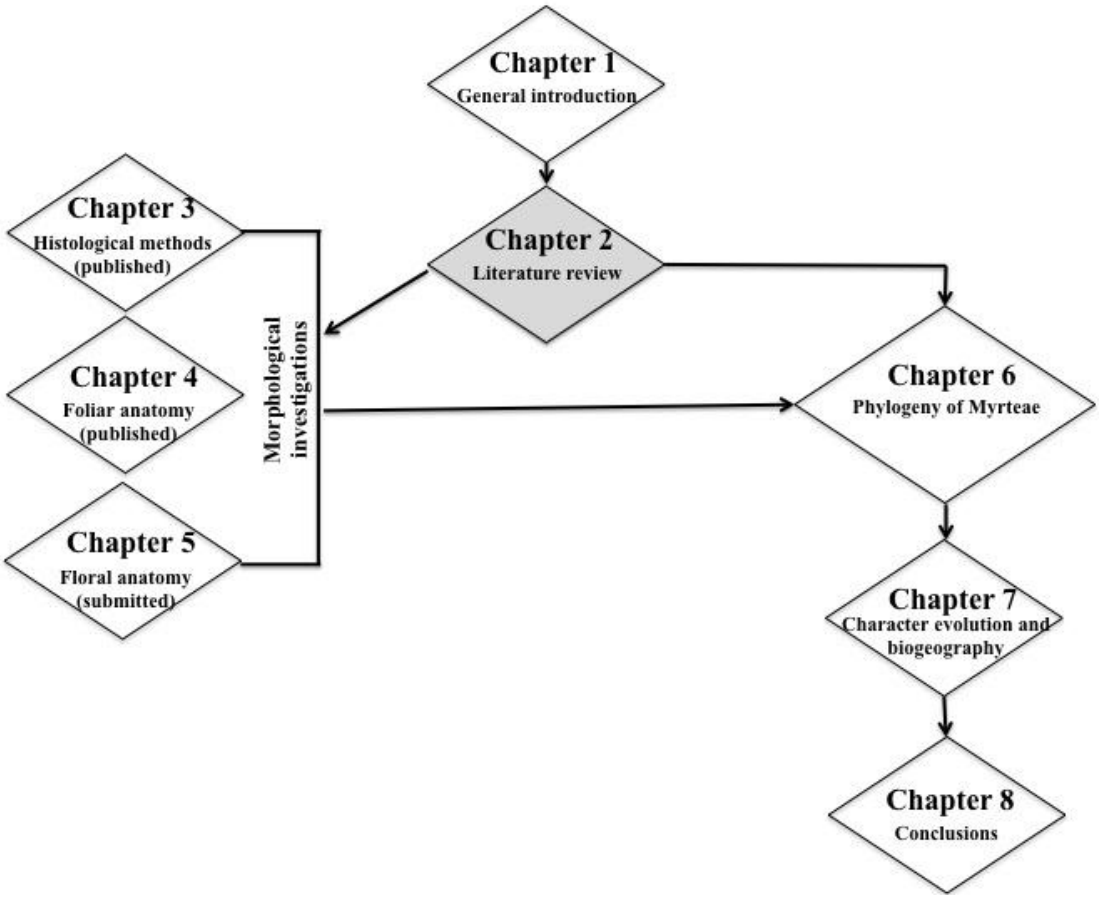
Chapter 6 is a phylogenetic study of the tribe Myrteae based on DNA sequences and morphological characters and includes all the species of Chilean Myrteae. This chapter addresses the third specific aim of this investigation.

Chapter 7 is a discussion on the evolution of morphological characters based on the phylogenetic results obtained in Chapter 6. In this chapter, the morphology (i.e., the leaf and flower anatomy/micromorphology) from non-Chilean species of Myrteae will be described and discussed along with the character evolution. Biogeographic distribution will be mapped onto the trees to reassess previous scenarios of ancestral area reconstruction. This chapter addresses the fourth specific aim of this thesis.

Chapter 8 outlines the main conclusions formulated from this research, drawing special attention to the contribution of this thesis to the existing scientific knowledge about the tribe Myrteae. Recommendations and guidelines for future work are provided in this chapter.

A flow chart that shows the connection between different chapters and the organization of the thesis will be shown at the start of each chapter. Figures and tables relevant to each section are placed after the conclusion section of that chapter. A single reference list compiled from

all chapters is presented after Chapter 8. Additional tables, figures and outcomes relevant to the PhD process (e.g., published journal articles, conference abstracts) have been placed in the appendices..


Chapter 1
General introduction
Chapter 2
Literature review
Chapter 3
Histological methods (published)
Chapter 4
Foliar anatomy (published)
Chapter 5
Floral anatomy (submitted)
Morphological investigations
Chapter 6
Phylogeny of Myrteae
Chapter 7
Character evolution and biogeography
Chapter 8
Conclusions

CHAPTER 2: Literature review

This chapter provides a comprehensive review of the existing taxonomic, phylogenetic, morphological and biogeographic literature regarding the tribe Myrteae with focus on the Chilean taxa. Herein, the knowledge gaps identified at the start of the PhD process will be highlighted.

2.1 Introduction

Myrtaceae Juss. is an angiosperm family of trees and shrubs in the order Myrtales (APG IV, 2016). It is divided into two subfamilies (Myrtoideae and Psiloxyloideae), 17 tribes and 142 genera (Wilson et al., 2005; Govaerts et al., 2008). The estimated number of species in the family ranges from 3,600 (Gadek et al., 1996), 4000 (Sytsma et al., 2004) to 5,500 (Govaerts et al., 2008; Biffin et al., 2010). The differences in the number of species are mainly attributed to the complex taxonomy of the family and new systematic investigations (Lucas et al., 2005). Myrtaceae is the eighth largest family of angiosperms in terms of number of species (Snow et al., 2012) and is regarded as economically important, as source of edible fruits, essential oils, wood and honey (Wilson, 2011). The family has a postulated origin in Gondwana (Johnson and Briggs, 1984; Sytsma et al., 2004) and is mainly distributed in the southern hemisphere, with a high diversity in South America, Central America and Australia (Ladiges et al., 2003). Areas with significantly fewer species of Myrtaceae are subequatorial Africa and adjacent Madagascar, the Mediterranean, China, India, Mexico and Florida (Wilson, 2011). The species occur in a wide range of habitats, including seasonally dry areas, coastal sands, rainforests, tropical moist forests, savannahs and mangroves (Biffin et al., 2010; Wilson, 2011). Species in this family share a number of morpho-anatomical characters, such as being woody, with entire, simple, evergreen and mostly opposite leaves, internal phloem in all organs and oil glands (Metcalfe and Chalk, 1979; Cronquist, 1988; Ciccarelli et al., 2008). Myrtaceae have polystemonous, mostly bisexual, actinomorphic flowers (Wilson, 2011), with partially inferior to fully inferior ovaries (Conti et al., 1997) and hypanthium with nectaries (Briggs and Johnson, 1979). The fruits are generally classified as either fleshy (berry) or dry (capsular), but other fruit types (drupes) also occur in this family (Wilson, 2011). Fruit consistency (fleshy versus dry) was widely used in the past to divide the family into subfamilies (Niedenzu, 1983).

Phylogenetic analyses have been critically important for the current systematics of angiosperms (APG IV, 2016; Soltis et al., 2005; Judd et al., 2008; Doyle, 2012), and they have largely contributed to recent taxonomic changes in Myrtaceae, at both family and genus level (Wilson et al., 2005; Craven and Biffin, 2010; Snow et al., 2011; Murillo et al., 2012; Mazine et al., 2014). The first classification of Myrtaceae using morphological phylogenetics resulted in an alliances-suballiances system, regarded as relatively equivalent to a formal tribes-subtribes system (Briggs and Johnson, 1979; Johnson and Briggs, 1984). A number of phylogenetic analyses (Johnson and Briggs, 1984; Gadek et al., 1996; Wilson et al., 2001, 2005) have indicated that fruit consistency is not a reliable character to delimitate tribes or subfamilies in Myrtaceae. Most of the fleshy-fruited species from Australasia (mainly the genus *Syzygium*) have been confirmed as a lineage independent to fleshy-fruited species from Central America, South America and eastern Australia (Johnson and Briggs, 1984; Gadek et al., 1996).

The chloroplast region *matK* has shown to be phylogenetically informative when analysing intergeneric relationships within Myrtaceae. The current classification of Myrtaceae (Wilson et al., 2005) is based on a molecular *matK* phylogeny, where the family is classified into 17 tribes in two subfamilies, namely Psiloxiloideae (2 tribes) and Myrtoideae (15 tribes) (Fig. 2.1). Relationships between the tribes have been recently investigated by Biffin et al. (2010) and Thornhill et al. (2015). The tribe Myrteae (subfamily Myrtoideae) currently encompasses all the American species of Myrtaceae (except for *Metrosideros stipularis*) and the fleshy-fruited species in Australasia other than the genus *Syzygium* (*sensu* Craven and Biffin, 2010) (Wilson et al., 2005).

The fleshy-fruited tribe Myrteae (*sensu* Wilson et al., 2005) is the richest tribe of Myrtaceae with ca. 2500 species in ca. 54 genera and includes most of the species previously considered as part of the subfamily Myrtoideae (Biffin et al., 2010). Myrteae is mainly distributed in Central and South America, with some genera in the Australasian region (Lucas et al., 2007) (Appendix 2). The tribe Myrteae is represented in Chile by 25 species in nine genera, of which 17 species are endemic to Chile (Landrum, 1988a). The species of Chilean Myrteae occur from the semi-arid centre to the temperate forests in the southern tip of the country and in the Juan Fernandez Archipelago (Landrum, 1988a; Murillo and Ruiz, 2011). Species mainly occur humid or flooded habitats, usually gullies (Kausel, 1942, 1956; Landrum, 1988a). Chilean Myrtaceae are an important component in the upper, middle and even lower strata of the forests of southern South America (Hildebrand-Vogel, 2002).

The focus of the present literature review will be the taxonomy, phylogeny, morphology/structure and biogeography of tribe Myrteae, with focus on the Chilean taxa. This review will not deal with the Chilean-Argentinean species *Metrosideros stipularis* (tribe Metrosiderae), as it is not included in the tribe Myrteae.

2.2 Literature review

2.2.1 Taxonomy and phylogeny of Myrteae

Throughout the taxonomic history of the family, fleshy-fruited species have been variously grouped as tribe, subfamily and family and treated as a lineage independent to dry-fruited species (Wilson et al., 2001). De Candolle (1828) considered fleshy-fruited Myrtaceae grouped as the tribe Myrteae. Niedenzu (1893) classified fleshy-fruited species of Myrtaceae as the subfamily Myrtoideae, which contained only the tribe Myrteae. Kausel (1956) proposed the fleshy-fruited species of the family as a separate family, the family Myrtaceae. The currently accepted tribal rank of Myrteae is based on phylogenetic analyses using molecular data (Wilson et al., 2005). The tribe Myrteae has been traditionally classified into three subtribes mainly based on embryo morphology, namely Myrciinae, Eugeniinae and Myrtinae (Berg, 1855, 1857, 1858). Berg's classification has been widely recognised and only recently debated by phylogenetic analyses (Lucas et al., 2007). Kausel (1956) classified the fleshy-fruited species (family Myrtaceae sensu Kausel, 1956) into five subfamilies mainly based on two types of seed germination (hypogeal and epigeal). McVaugh (1968) divided the tribe Myrteae into six "informal groups" based on a number of morphological characters.

Taxonomic classifications of Myrteae have included the species of Chilean Myrteae in a number of different groups (Table 2.1). McVaugh (1968) did not attempt to classify five genera of Chilean Myrteae, for possessing atypical combination of characters and unusual distribution patterns (Landrum 1986) (Table 2.1). Atypical genera, namely *Legrandia*, *Luma*, *Myrceugenia*, *Blepharocalyx* and *Myrcianthes* have been called "evolutionary experiments" (McVaugh, 1968), because they either show unique features or distinct combinations of otherwise common features (e.g. number of calyx lobes, inflorescence type and embryo type). Johnson and Briggs (1984) classified the Chilean species in the *Myrtus* alliance (Myrtinae), the *Eugenia* alliance (Eugeniinae) and the *Myrcia* alliance (Myrciinae) (Table 2.1). The last comprehensive taxonomic revision of Myrteae in Chile recognises 25 species in eight genera for the tribe (Landrum, 1988a). The revalidation of *Nothomyrcia fernandeziana* (Juan Fernandez Archipelago) as a valid name over *Myrceugenia fernandeziana* based on

morphological and DNA sequences (Murillo and Ruiz, 2011; Murillo et al., 2012), updated the number to 25 species in nine genera for Myrteae. *Nothomyrcia fernandeziana* has been phylogenetically related to *Blepharocalyx* (Murillo and Ruiz, 2011; Murillo et al., 2012, 2013). Some genera of Myrteae with species occurring in Chile have been subject of specific taxonomic revisions, namely *Amomyrtus* (Landrum and Salywon, 2004), *Myrceugenia* (Landrum, 1981b), *Myrteola* (Landrum, 1988b) and *Myrcianthes* (Landrum and Grifo, 1988).

The tribe Myrteae is statistically well suported as a monophyletic group (Murillo et al., 2013; Lucas et al., 2007) and closely related to other five other tribes in the subfamily Myrtoideae, namely Syzygieae, Backhousieae, Metrosidereae, Tristanieae and Kanieae (Biffin et al., 2010; Thornhill et al., 2015) (Fig 2.1). Internal relationships in Myrteae are not completely clear and most clades are not well supported (Wilson et al., 2005; Lucas et al., 2007; Biffin et al., 2010; Murillo et al., 2013). Suprageneric relationships in Myrteae have shown the genus *Myrtus* (Mediterranean) as sister to all the tribe Myrteae, based on nuclear (ITS, ETS) and chloroplast (*matK*, *psbA-trnH*, *ndhF*) DNA sequences (Sytsma et al., 2004; Lucas et al., 2007; Biffin et al., 2010; Murillo et al., 2013). The number of clades identified in Myrteae varies from six (Murillo et al., 2013) to seven (Lucas et al., 2007). An Australasian clade consisting of *Decaspermum*, *Rhodamnia*, *Gossia*, *Austromyrtus* and *Rhodomyrtus* has high statistical support in most of analyses. Major relationships in Myrteae include a number of clades with mainly Brazilian genera such as the "*Myrcia* group", "*Eugenia* group" and "*Plinia* group". The "*Myrcia* group" includes *Myrcia*, *Marlierea*, *Calyptranthes*, *Gomidesia* (Lucas et al., 2007) and *Algrizea* in Murillo et al. (2013), while the "*Eugenia* group" includes only *Eugenia* and *Myrcianthes*. The "*Plinia* group" is a small clade that encompasses *Plinia*, *Siphoneugena*, *Neomitranthes*, *Myrciaria* (Murillo et al., 2013) and *Algrizea* (Lucas et al., 2007).

Various studies have placed Chilean Myrteae in at least three clades, but with some major differences (Fig. 2.2), possibly due to differences in taxonomic sampling and selection of molecular loci. Within the different clades of Myrteae, the Chilean Myrteae are often the sister group to the eastern South American or Australasian genera in that clade (Lucas et al., 2007; Murillo et al., 2013). The genera *Blepharocalyx*, *Luma* and *Myrceugenia* have been grouped in the "*Myrceugenia* group" (Lucas et al., 2007), which is sister to the larger "Myrcia group" and "Plinia group". Relationships between *Blepharocalyx*, *Luma* and *Myrceugenia* have been previously supported in earlier morpho-anatomical studies (e.g., presence of tracheids; scalariform perforation plates, Schmid, 1984). In further investigations,

Luma and *Blepharocalyx* were proposed as two distantly related lineages (Murillo et al., 2013) with *Luma* sister to all Myrteae, except for *Myrtus communis*. A phylogeny of *Myrceugenia* has been conducted based on 38 morphological characters to explain the disjunction of the genus (c. 40 species) between Chile (14 species) and Brazil (26 species) (Landrum, 1981a). *Myrceugenia* was monophyletic and considered a 'primitive' genus of Myrteae and Brazilian and Chilean species were grouped in different clades without a clear differentiation (Landrum, 1981a). However, more recent studies based on molecular data, have indicated that Chilean species of *Myrceugenia* form three to four separate lineages, whereas Brazilian species are grouped in one clade and *M. rufa* is sister to all species of the genus (PP 1.0; Murillo et al., 2013). Murillo et al. (2016) reconstructed the phylogeny of *Myrceugenia* and indicated that *M. rufa* is not sister to the genus, but it is nested in a clade with other four Chilean species, namely *M. correifolia*, *M. lanceolata*, *M. obtusa* and *M. exsucca*. In the former study, the phylogeny of Myrteae was also reconstructed, but only based on two nuclear markers (ITS, ETS) and generally weakly supported.

Ugni molinae and *Myrteola nummularia* have been included in the "*Myrteola* group", along with the New Zealand genera *Lophomyrtus* and *Neomyrtus* (Lucas et al., 2007). Other phylogenetic analyses have placed *Myrteola* in a group with *Pimenta* and *Campomanesia* (Biffin et al., 2010) or closely related to the Australasian *Rhodamnia* and *Decaspermum* (Murillo et al., 2013) (Fig. 2.2). Phylogenetic trees in Murillo et al. (2013) exclude *Myrteola nummularia* from the "*Myrteola* group", and indicate that the species might be closely related to either *Myrceugenia* or the group formed by *Decaspermum* and *Rhodamnia* (Australasia). Landrum (1988b) suggested that *Myrteola* might be related to *Myrtus communis*. Phylogenetic analyses have nested *Ugni* in the same clade as *Pimenta* and *Campomanesia* (Biffin et al. 2010). *Ugni selkirkii* was indicated as sister to *Myrceugenia* in Murillo et al., (2013). Since *Ugni candollei* and *Ugni myricoides* (Central and South America) have never been included in a phylogenetic study, the monophyly and systematic position of *Ugni* (4 species) in unclear. *Amomyrtus* and *Legrandia* form a monophyletic group sister to a clade formed by *Pimenta, Acca, Psidium* and *Campomanesia* in the "*Pimenta* group" (Lucas et al., 2007, Murillo et al., 2013) (Fig. 2.2). *Amomyrtus*, *Legrandia*, and *Pimenta* have shown variable systematic positions depending on the phylogenetic method and genomic region (Murillo et al., 2013, 2016). In Biffin et al. (2010) and based on ITS, *matK* and *ndhF* DNA sequences, the systematic position of *Amomyrtus* is unresolved in a clade including the *Pimenta* and *Eugenia* groups.

However, when examined closely, many of the relationships discussed in previous studies, are not statistically well supported. The sister positions of Chilean Myrteae to other major clades needs to be investigated further by more comprehensive sampling and inclusion of additional data that will provide robust statistical support in order to discuss biogeographic implications of these relationships. The monophyly and systematic position of the genus *Blepharocalyx* (three species) has not been corroborated, as *B. eggersii* (South America) has never been included in a phylogenetic analysis. *Myrcianthes coquimbensis* (north-central Chile) is the only species of the genus with a disjunct distribution and is yet to be included in a phylogenetic analysis. Molecular studies in Myrteae acknowledge that most of the subtribes or groups in their studies have low support and low resolution (Lucas et al., 2007; Biffin et al., 2010; Murillo et al., 2012, 2013, 2016). The inconsistency of results regarding some genera, suggest that more comprehensive sampling is required in order to understand the relationships of these genera (Murillo et al., 2013). Increased taxon sampling (e.g., *Myrcianthes coquimbensis, Ugni candollei, Blepharocalyx eggersii*), and new data sources such as morpho-anatomical characters, may elucidate the systematics of these groups.

2.2.2 Morphological characters in Myrteae

Morphological and anatomical characters have shown to be taxonomically and ecologically significant for a number of Myrteae. Myrteae possess indehiscent fleshy fruits, simple or dibrachiate hairs and transeptal vascularization of the ovary (Schmid, 1972, 1980; Wilson, 2011). Embryological characters have been relevant for the historical classification of the tribe (Wilson, 2011). A. P. de Candolle (1828) recognized three different embryo types within Myrteae and Berg (1855) divided the tribe into three subtribes based on these embryonic characters. Species with a well-developed hypocotyl and narrow-tiny cotyledons were grouped in the subtribe Myrtinae. Taxa that possessed thick and fleshy cotyledons and a relatively insignificant hypocotyl were considered as part of the subtribe Eugeniinae. Lastly, species with broad, thin and leafy cotyledons, folded into a bundle and having a horseshoe hypocotyl were considered part of Myrciinae (Berg, 1855). These three types of embryo were regarded as taxonomically useful for Myrteae, for the assumption that there is no tendency for one type to evolve into another (Landrum and Stevenson, 1986). Extensive embryological work has indicated that the tripartite classification in Myrteae according to embryo should be abandoned, due to several other character states that can be found (Snow et al., 2003).

Metcalfe and Chalk (1979), Schmid (1972, 1980, 1884) and Keating (1984) described morphological and anatomical diagnostic characters for several species and genera in the tribe. While molecular phylogenetics has been important for the intratribal classification of Myrteae, morphological characters have been widely used to support/contradict phylogenetic relationships (Lucas et al., 2011; Snow et al., 2011). For instance, phylogenetic studies of the large South American genus *Myrcia* and the pantropical genus *Eugenia* have shown clades supported by morphological features of flowers, inflorescences, bracteoles and placentation, (Lucas et al., 2011; Mazine et al., 2014). The Australasian genus *Gossia* was divided into three genera (*Austromyrtus*, *Lenwebbia*, *Gossia*) based on an extensive reassessment of morphology of the fruit, embryo, essential oils and ovary (Snow et al., 2003). The Australian *Lithomyrtus* is a little-known genus of shrubs and small trees with vegetative characters that resemble species adapted to arid environments (Wilson, 2011). *Lithomyrtus* is mainly distributed in coastal sands of northern Queensland and Northern Territory and has never been included in a phylogenetic analysis. A phylogenetic analysis with a more comprehensive sampling of *Austromyrtus*, *Lenwebbia*, *Gossia* and *Lithomyrtus* would clarify the evolutionary relationships of the Australasian Myrteae.

Along with gross morphology, micromorphological and anatomical characters have shown to be informative to distinguish genera and species of Myrteae at both suprageneric and infrageneric levels (Fontenelle et al., 1994; Cardoso et al., 2009; Gomes et al., 2009; Retamales and Scharaschkin, 2015). Leaf micromorphology (using SEM and LM) has been mainly studied in *Eugenia*, and some characters (e.g., paracytic stomata in South American species) have shown to be informative for distinguishing species from different geographic areas (Fontenelle et al., 1994; Haron and Moore, 1996). The use of anatomical characters has been regarded as a suitable approach to resolve taxonomical problems in some groups of Myrteae (Schmid, 1972, 1980; Cardoso et al., 2009; Gomes et al., 2009). The Australasian *Syzygium* (tribe Syzygieae) and the pantropical *Eugenia* once considered controversial genera with unclear generic limits, were consistently delimitated using floral anatomical characters, mainly type of ovular vascular supply (Schmid, 1972). Further anatomical characters used in Myrteae to distinguish this tribe from others include placentation (mainly axile in Myrteae) and number of ovules per ovary (Schmid, 1980). Cardoso et al. (2009) and Gomes et al. (2009) conducted studies on the leaf anatomy of many South American species of Myrteae and indicated that anatomical characters, alongside gross morphological features, can be used to diagnose species and genera. The main characters studied by Cardoso et al. (2009) and

Gomes et al. (2009) include presence of hypodermis, shape of midrib, confluence of leaf adaxial and abaxial phloem and sclerenchyma surrounding the midrib. Morpho-anatomical characters in cultivated species of Myrteae (e.g., *Myrtus communis*, *Psidium guajava*) must be treated with caution to establish character states, since variations might occur as product of altered phenotypic plasticity (Snow et al., 2003). In addition, histochemical observations (e.g., polyphenols in the cuticle, tannin and mucilage content in the mesophyll) in different species have been proposed as potentially taxonomically informative (Schmid, 1972; Schmid, 1980), but not tested in the phylogenetic context of Myrteae. The study of histochemistry (and anatomy) in plants is regarded as complex when taxa are rich in secondary compounds and phytochemicals (Evert and Eichhorn, 2006). Since Myrtaceae is well known for having phytochemicals and secondary compounds, the elaboration of a specific histochemical-anatomical protocol might improve the identification of these substances.

Character evolution studies are scarce in Myrteae, with only a limited number of morphological and anatomical characters investigated. Lucas et al. (2007) traced the evolution of four morpho-anatomical characters in Myrteae, namely placentation, embryo type, number of ovules and type of vessel perforations. These four characters have been shown to be homoplasious, but combinations of characters have been recognized as useful for clade diagnosis in Myrteae (Lucas et al., 2007). The evolution of pollen characters has been comprehensively assessed for Myrtaceae and Myrteae, but most characters have been assessed as being homoplasious (e.g., pollen width, exine pattern morphology) (Thornhill et al., 2012b, 2012c, 2012d, 2012e). A few gynoecium characters (e.g., number of receptacular bundles, placenta vasculature) are considered potentially informative in studying the evolution of Myrteae (Pimentel et al., 2014). Stamen position is a phylogenetically informative character, since erect stamens are present in most clades of the tribe, while semi-curved and strongly curved patterns are potential synapomorphies for other clades (Vasconcelos et al., 2015). A comprehensive character evolution study based on a wide range of characters has not been conducted in Myrteae.

Taxonomic revisions on Chilean Myrteae have strongly relied on a number of gross morphological characters to delimit genera and interpret evolutionary affinities (Reiche, 1897; Kausel, 1942b, 1947, 1956; McVaugh, 1956; Landrum, 1988a). Morphological characters that have been considered important for classifying Chilean Myrteae include type of inflorescence, type of hairs, number of sepals and petals, caducity of bracteoles and type of embryo (Landrum, 1988a). On the basis of type of embryo, Chilean genera have been

previously included in the three historical subtribes of Myrteae (Myrciinae, Myrtinae, Eugeniinae). *Luma* has a fourth type of embryo, which is in between that of the type seen in Myrciinae and Eugeniinae. It is hypothesized that these different types of embryo evolved independently from a Myrteae ancestor with an "*Ugni*-like" reduced embryo (Landrum, 1988a; Lucas et al. 2007). *Luma* has been considered part of a fourth independent evolutionary lineage or a genus resembling Myrciinae or Eugeniinae (Landrum, 1986). Based on embryological and inflorescence characters, genera with Chilean representatives have been regarded as the primitive form within each recognised subtribe, for instance *Blepharocalyx* in Myrtinae, *Myrcianthes* in Eugeniinae and *Myrceugenia* in Myrciinae (McVaugh, 1968; Landrum, 1981b).

The anatomy of the tribe Myrteae has not been documented in detail (P.G. Wilson, pers. comm.) and there is no a comprehensive anatomical study of the Chilean Myrteae. The majority of available anatomical information for Chilean species of Myrteae is related to wood anatomy (Ragonese, 1976; Landrum, 1981b; Schmid and Baas, 1984; Wilson et al., 2001). Most of the genera of Chilean Myrtaceae possess wood anatomical character states considered to be primitive. Some of these features are scalariform perforation plates in some species of *Myrteola*, *Myrceugenia*, *Luma* and *Ugni* and helical wall thickenings in *Myrceugenia* (Schmid and Baas, 1984; Landrum, 1981b). Scalariform perforated plates in vessels have been attributed to ancient species (Stern, 1978), being supposedly an adaptation to cooler or mountain environments (Jansen et al., 2004). Other wood characters include tracheids in *Luma, Blepharocalyx* and *Amomyrtus* (Ragonese, 1976). The leaf anatomy of the Chilean Myrteae had never been studied in detail until the beginning of this investigation.

Leaf anatomy has been regarded as potentially helpful for delimitation of genera in Myrteae, specially combined with gross morphological characters (Cardoso et al., 2009; Gomes et al., 2009). Flower anatomy has never been studied comprehensively in any species of Chilean Myrteae, other than *Ugni molinae* (Pimentel et al., 2014), which is a well-known cultivated species around the world (Aguirre et al., 2006). Comprehensive anatomical investigations of the leaf and flower anatomy of Chilean Myrteae may provide relevant information for phylogenetic analyses and character evolution.

2.2.3 Historical biogeography of Myrteae

Geographic distribution of extant taxa

The monophyletic tribe Myrteae (*sensu* Wilson et al., 2005) comprises ca. 2500 species and is mainly distributed in South America, Central America (Lucas et al., 2007) and Australasia (Wilson, 2011). The tribe Myrteae encompasses most of the rainforest species of Myrtaceae occurring in Australasia and nearly all the South American species of the family (Biffin et al., 2010) (Appendix 2). The South American genera *Acca, Myrcia, Eugenia, Calyptranthes, Pimenta, Psidium, Siphoneugena* and *Gomidesia* represent nearly the 70% of the diversity of the tribe (Govaerts et al., 2008). In Australasia, *Austromyrtus*, *Decaspermum*, *Gossia, Lenwebbia, Rhodomyrtus, Rhodamnia* are mainly distributed in rainforests in Eastern Australia, China and the Pacific Islands (Appendix 2). Nearly all the Chilean Myrteae occur in the "Chilean Winter Rainfall-Valdivian Forest Hotspot", an area located in between 25º and 47º south latitude. This region that includes humid temperate forests, shrublands and riparian habitats, is known for a high level of plant endemism (Arroyo et al., 2006). This area is considered a priority for plant conservation at global scale (Myers et al., 2000). This biogeographic region includes the Juan Fernandez Archipelago, where three species of Myrteae are endemic, namely *Myrceugenia schulzei*, *Nothomyrcia fernandeziana* and *Ugni selkirkii* (Ruiz et al., 1994). Four genera (*Amomyrtus*, *Legrandia*, *Luma* and *Nothomyrcia*) are endemic to the humid temperate forests in Chile and Argentina. *Amomyrtus* and *Luma* possess two species each, while *Legrandia* and *Nothomyrcia* are monospecific genera (Landrum, 1988a). *Nothomyrcia* is endemic to the Robinson Crusoe Island, Juan Fernandez Archipelago (Murillo and Ruiz, 2011). The remaining five genera have a more extensive distribution range and also occur outside of Chilean-Argentinean forests. The genus *Ugni* has four species, two of which occur in the forests of mainland Chile, one is endemic to the Juan Fernandez Archipelago and one occurs in Mexico and Central America (Wilson, 2011). *Blepharocalyx* possesses three species, one of which occurs in Chile and the remaining occur in the Caribbean islands, Brazil, Paraguay, Uruguay and Argentina. *Myrcianthes* has around 30 species, with one species in Chile and the remaining mainly distributed from Mexico to Peru (Wilson, 2011). *Myrteola* has three species, of which one occurs in Chile, Peru, Argentina, Venezuela and Colombia, while the other two species occur in Colombia and Venezuela (Landrum, 1986, 1988b). The genus *Myrceugenia* comprises around 40 species, of which nine occur exclusively in continental Chile, three species (and one variety) occur in

central-southern Chile and Argentina, one species is endemic to the Juan Fernandez Islands and 17 species are distributed in southeast Brazil (Landrum, 1981b) (Appendix 2).

Chile is a South American country with particular natural barriers, which have influenced the geographic seclusion of its flora (Moreira-Muñoz, 2011). Chilean forests and shrublands are established in a restricted vegetational and climatic area, delimited by deserts in the north, the Andes by the east and the Pacific Ocean by the south and west (Landrum, 1988a; Arroyo et al., 2006). This condition has enabled the evolution of several endemic plant lineages (e.g., Gomortegaceae, *Legrandia*, *Neoporteria* (Cactaceae), *Pitavia* (Rutaceae)) (Moreira-Muñoz, 2011). According to Marticorena (1990), 47% of the Chilean flora is endemic to Chile, which is the highest concentration of endemic plants in a South American country. The majority of the species of Myrteae occurring in Chile are endemic (Zuloaga et al., 2008). Most of these species have a very restricted distribution and an endangered conservation status (Benoit, 1989; IUCN, 2012). An arid zone called "Arid Diagonal of South America" crosses South America from the southeast to the northwest (Villagrán and Hinojosa, 1997) and separates the southern Chilean-Argentinean forests from the forests in the north of the continent (Brazil, Bolivia, Peru). These particular geographical frontiers may be responsible for the isolation, and subsequent evolutionary history, of a number of plant lineages, included Myrtaceae (Villagrán and Hinojosa, 1997; Moreira-Muñoz, 2011).

Fossils and historical distribution

Fossils early suggested the high affinity of the floras between South America and Australasia due to Antarctic connections, which also explains the taxonomic diversity of Myrtaceae in these two areas (Burbidge, 1960). During the Eocene, Southern South America was mainly occupied by a mixed tropical-subantarctic palaeoflora (e.g., Nothofagaceae, Proteaceae) with the existence of a belt of tropical flora, including Lauraceae and Myrtaceae (Moreira-Muñoz, 2011). The geological period of separation of South America from Antarctica and separation of Australia from Antarctica is controversial, since could have occurred from the Late Jurassic to the Paleocene or Eocene (Orme, 2007). Fossils of *Eucalyptus* from early Eocene found in Patagonia are the oldest register for the genus and confirm the past presence of the Australasian genus in South America (Hermsen et al., 2012). Some taxa, such as the South American *Metrosideros stipularis* might be product of the connections or propinquity until mid-Tertiary in the Antarctic region (Johnson and Briggs, 1984). The earliest fossil record attributed to Myrtaceae is *Myrtaceidites* pollen from the Late Cretaceous of Gabon (Africa),

Borneo and Australia (100-80 mya) (Boltenhagen, 1976). Extant Myrtaceae pollen is very distinctive from other families, but it has a consistent morphology within the family, which makes problematic to attribute fossil pollen to ancestors of certain extant lineages (Pigg et al., 1993; Wilson, 2011). Fossil leaves of Myrtaceae from the Early Paleogene of Antarctica, New Zealand, Australia, South America and North America suggest the widespread distribution of the family during the Eocene (Wilson, 2011).

The fossil record of Myrteae has been helpful to infer the origin and past distributions of the tribe. Berry (1915) reviewed the fossils of Myrteae investigated to that date, with special reference to those described in the northern hemisphere as ancestors of *Myrcia* and *Eugenia* from the Cretaceous, which confirms the broader past distribution of a number of extant Australasian and South American genera of Myrteae. The presence of the fossil species *Paleomyrtinaea princetonensis* from the mid-Eocene Princeton Chert of British Columbia (Canada) and the Paleocene of North Dakota (United States) (Pigg et al., 1993), suggests the extended distribution of Myrteae due to warmer conditions during these two periods (Lucas et al., 2007). The fossil record of *Paleomyrtinaea princetonensis* was considered the first occurrence of myrtaceous fleshy fruits in North America (Pigg et al., 1993).

Pigg et al. (1993) proposed that the early radiation of tribe the Myrteae took place during the late Cretaceous of North America, based on the fossil fruits of *Paleomyrtinaea princetonensis.* Sytsma et al. (2004) and Biffin et al. (2010) indicated that the age of Myrteae might be 50-55 mya. However, fossils of *Myrceugenelloxylon antarcticus* from the Late Cretaceous (68 mya) to the mid-Eocene of Antarctica (Poole et al., 2001) suggest that the tribe Myrteae might be older than previously proposed. The wood anatomy of *Myrceugenelloxylon antarcticus* resembles *Luma apiculata* (Poole et al., 2001), a species considered plesiomorphic and sister to the rest of the group (McVaugh, 1956). The earliest fossil records of Myrtaceae in southern South America-Antarctica are from 80-70 mya (Late Cretaceous) and are mainly assigned to *Eugenia* and *Luma* (Poole and Cantrill, 2006). The ancestral area of the tribe Myrteae is not completely clear, as biogeographical analyses are scarce. Although migration events (dispersals) largely explain the relationships of floras in the southern hemisphere (Skottsberg, 1956; Sytsma et al., 2004), continental drift has been considered the main mechanism to explain the presence of fleshy-fruited Myrtaceae in both Australasia and the Americas (Johnson and Briggs, 1984). Dispersal-Vicariance analyses in Lucas et al. (2007) indicated the origin of the tribe Myrteae in Gondwana with posterior migration to South America. Lucas et al. (2007) proposed that the radiation of Myrteae

started South America followed by colonization via the Andes. According to Lucas et al. (2007), ancestors of extant genera of Myrteae originated in the south of South America and dispersed towards the north of the continent (most of the Myrteae genera), South East Asia, Africa (*Eugenia*) and New Zealand (*Lophomyrtus* and *Neomyrtus*).

Myrteae has been mentioned as part of the Palaeocene flora of Central and Southern Chile, mainly as azonal vegetation in higher areas (Romero, 1993). Fossils of Myrtaceae suggest the widespread presence of the family along with conifers and *Nothofagus* species in Antarctica (warm cool temperate biome) during the early Palaeocene (55 mya) (Moreira-Muñoz, 2011; Truswell, 1990). Ancestors of current species of Chilean Myrteae associated with sclerophyll vegetation were established under warm and humid climate conditions during the mid Miocene (20-15 mya), due to the incipient Andes that provided a rain shadow effect (Hinojosa et al., 2006). Quaternary glaciations during the Pleistocene affected the replacement of Chilean flora as a whole, including species of Myrteae in the Southern Andes and Central Chilean Andes vegetation (Villagrán et al., 1998; Hinojosa et al., 2006, Moreira-Muñoz, 2011).

The high number of genera compared to the accepted number of species of Chilean Myrtaceae could be related to the early isolation of Chilean flora (Villagrán and Hinojosa, 1997), which took place approximately 10-30 mya during the Eocene-Miocene (Landrum, 1981a). Concentration of endemic and monotypic genera of Myrtaceae and other families in Central-Southern Chile might be a result of refuges during Pleistocene glaciations and repeated cycles of isolation due to advance of the glaciers (Moreira-Muñoz, 2011). Evolution of species considered atypical (*Legrandia, Luma, Myrceugenia, Blepharocalyx, Myrcianthes*) could have been influenced by the geographical isolation of this area since the Miocene (McVaugh, 1968; Landrum, 1981a). These apparently ancient Myrtaceae genera are mainly distributed in Central-South Chile, some of them with a minor presence in the Argentinean Andes (Landrum, 1988a). It has been suggested that ancestors of the New Zealand genera (*Lophomyrtus, Neomyrtus*) originated in western South America, considered the area of origin of the "*Myrteola* group" (Lucas et al., 2007). Murillo et al. (2012) postulated the origin of *Myrceugenia* in southern South America, followed by dispersal to Brazil and to the Juan Fernandez Islands. Murillo et al. (2012) hypothesized that ancestral species of *Myrceugenia* dispersed from southern to northern Chile between the early and mid-Miocene and then to Brazil approximately 10-15 mya. The formation of the arid diagonal of South America, influenced by the presence of the Paranaense Sea (15 mya) and the Andes uplift during the

Pliocene, separated northern from southern South America and contributed to the disjunction of Chilean and Brazilian *Myrceugenia* (Murillo et al., 2012). This hypothesis contrasts with previous analyses that indicated *Myrceugenia* used to occur continuously across South America and became divided (Landrum, 1981a). Murillo et al. (2016) indicated that *Myrceugenia* diverged into four different lineages during the early Miocene, three of them evolving in Chile with a number of dispersal events to the north and south and to the Juan Fernandez Islands. *Myrceugenia* fossils from Chile include leaves from the early Eocene (30 mya) and early Miocene (21 mya) of Central Chile (Gayo et al., 2005). These and other fossil records described from the Argentinean Paleocene-Eocene indicate that *Myrceugenia* species were already widely distributed in the early Paleocene (60 mya) (Landrum, 1981a). Geological events, such as the formation of the arid diagonal and the uplift of the Andes contributed to the confinement of many species of *Myrceugenia* in south-central Chile, while other species were restricted to southeastern Brazil (Landrum, 1981a).

Divergence ages

The divergence age between South American and Australasian Myrteae has been estimated as 43.9 (39.1-50.1) mya and explained as product of vicariance (Thornhill et al., 2015). However, estimated divergence ages do not overlap with the separation of Australia, Antarctica and South America (35-28 mya, McLoughlin, 2001) (Thornhill et al., 2015). The divergence between the South American *Myrteola* and New Zealand species has been estimated as 23.6 (23-25.5) mya explained by long distance dispersal (Thornhill et al., 2015). The presence of *Myrtus* as the only Myrteae genus in the northern hemisphere (estimated 50-53 mya, Sytsma et al. 2004) could have more than one interpretation (Thornhill et al., 2015). Whether the presence of *Myrtus* in Europe is the result of one single long distance dispersal from East Gondwana or the sole remaining of a past widespread Laurasian clade sister group to the Southern Myrteae, is unclear (Thornhill et al., 2015). The systematic position of *Myrtus* needs further investigation, since many analyses have shown only weak to moderate support for relationships within genus (Sytsma et al., 2004; Lucas et al., 2007; Murillo et al., 2013). Biogeographic implications for the tribe strongly depend on more accurate estimations of the sister group relationships of *Myrtus* within Myrteae. Murillo et al. (2016) suggested that the estimated age of divergence of the tribe Myrteae might be older than proposed (75-90 mya) and indicated that this age is consistent with the fossil record in South America, which is consistent with the results of Poole et al. (2001) In the former study, the fossils

Myrceugenelloxylon antarcticus and *Myrceugenia chubutense* were used to calibrate the nodes of the ancestors of *Myrceugenia* and *Luma*, due to anatomical similarities with the wood of these genera. However, more anatomical evidence on extant species might be necessary in order to assign fossils more accurately to the nodes.

2.3 Conclusion

This chapter has reviewed the main taxonomic, phylogenetic, anatomical and biogeographic studies completed on Myrteae, particularly those including Chilean species. Chilean Myrteae have been highlighted as phylogenetically and biogeographically relevant in the South American-Australasian context, since many species have a sister group relationship to New Zealand and South American clades. Some Chilean Myrteae that need further study (*Legrandia, Luma, Myrceugenia, Blepharocalyx, Ugni*) are considered atypical in the family for having unique features, unusual distribution patterns or a particular combination of characters. The systematic position of all Chilean Myrteae has not been determined, since *Myrcianthes coquimbensis* and *Ugni candollei* have not been included in any phylogenetic analysis. The monophyly of the small genera *Ugni* (four species) and *Blepharocalyx* (three species) has never been tested. The inconsistency of results regarding some genera between previous studies, indicates that more comprehensive sampling is required in order to understand the relationships of these genera (Murillo et al. 2013, 2016). Similarly, the understanding of the relationships within Myrteae might be improved if further genera are included (e.g., *Lenwebbia*, *Austromyrtus*, *Lithomyrtus*). Most of the phylogenetic analyses to date in Myrteae are entirely based on DNA sequences while anatomical and histochemical characters have been underused as sources of data, particularly in those studies including Chilean genera. In order to reliably describe histochemical characters, an optimized protocol for Myrtaceae is yet to be developed. A comprehensive anatomical investigation of leaves and flowers in Chilean Myrteae may help to resolve the phylogeny of Myrteae and together with known characters, form the basis for a character evolution analysis. Results from a robust and well supported phylogeny might be the basis for reconstruction of ancestral areas in Myrteae and specifically for Chilean genera, given the complex geologic and biogeographic history of Chile and South America.

Table 2.1. Historical taxonomy and informal groups from phylogenetic analyses of the Chilean Myrtaceae.

Taxon	Taxonomic classification or phylogenetic group according to:				
	Berg (1955)	McVaugh (1968)	Briggs and Johnson (1979) alliances	Lucas et al. (2007)	Murillo et al. (2013)
Amomyrtus luma (Molina) D. Legrand and Kausel	Myrtinae (as *Myrtus*)	Uncertain	*Myrtus*	*Pimenta* group	*Pimenta* group
Amomyrtus meli (Phil.) D. Legrand and Kausel	Myrtinae (as *Myrtus*)	Uncertain	*Myrtus*	Pimenta group	Not included in study
Blepharocalyx cruckshanksii (Hook. and Arn.) Nied.	Myrtinae (as *Temu*)	Myrtinae (as *Temu*)	*Cryptorhiza*	*Myrceugenia* group	*Blepharocalyx-M. fernandeziana* group
Legrandia concinna (Phil.) Kausel	Myrtinae	Uncertain	*Cryptorhiza*	*Pimenta* group	Pimenta group
Luma apiculata (DC.) Burret	Uncertain	Uncertain	*Eugenia*	*Myrceugenia* group	*Luma* group ?
Luma chequen (Feuillée ex Molina) A. Gray	Uncertain	Uncertain	*Eugenia*	*Myrceugenia* group	*Luma* group ?
Myrceugenia chrysocarpa (O. Berg) Kausel	Myrciinae	Uncertain	*Myrcia*	Not included in study	Chilean *Myrceugenia*
Myrceugenia colchaguensis (Phil.) Navas	Myrciinae	Uncertain	*Myrcia*	Not included in study	Chilean *Myrceugenia*
Myrceugenia correifolia (Hook. and Arn.) O. Berg	Myrciinae	Uncertain	*Myrcia*	Not included in study	Chilean *Myrceugenia*
Myrceugenia exsucca (DC.) O. Berg	Myrciinae	Uncertain	*Myrcia*	Not included in study	Chilean *Myrceugenia*
Myrceugenia lanceolata (Juss. ex J. St.-Hil.) Kausel	Myrciinae	Uncertain	*Myrcia*	*Myrceugenia* group	Chilean *Myrceugenia*

Myrceugenia leptospermoides (DC.) Kausel	Myrciinae	Uncertain	*Myrcia*	*Myrceugenia* group	Chilean *Myrceugenia*
Myrceugenia obtusa (DC.) O. Berg	Myrciinae	Uncertain	*Myrcia*	Not included in study	Chilean *Myrceugenia*
Myrceugenia ovata (Hook. and Arn.) O. Berg	Myrciinae	Uncertain	*Myrcia*	*Myrceugenia* group	Chilean *Myrceugenia*
*Myrceugenia ovata var. nano*phylla (Burret) L.R. Landrum	Myrciinae	Uncertain	*Myrcia*	Not included in study	Chilean *Myrceugenia*
Myrceugenia parvifolia (DC.) Kausel	Myrciinae	Uncertain	*Myrcia*	Not included in study	Chilean *Myrceugenia*
Myrceugenia pinifolia (F. Phil.) Kausel	Myrciinae	Uncertain	*Myrcia*	Not included in study	Chilean *Myrceugenia*
Myrceugenia planipes (Hook. and Arn.) O. Berg	Myrciinae	Uncertain	*Myrcia*	Myrceugenia group	Chilean *Myrceugenia*
Myrceugenia rufa (Colla) Skottsb. ex Kausel	Myrciinae	Uncertain	*Myrcia*	Not included in study	Chilean *Myrceugenia*
Myrceugenia schulzei Johow	Myrciinae	Uncertain	*Myrcia*	Not included in study	Chilean *Myrceugenia*
Myrcianthes coquimbensis (Barnéoud) L.R. Landrum and Grifo	Eugeniinae	Eugeniinae	*Eugenia*	Not included in study	Not included in study
Myrteola nummularia (Poir.) O. Berg	Myrtinae	Myrtinae	*Myrtus*	*Myrteola* group	Not grouped
Nothomyrcia fernandeziana (Hook. and Arn.) Kausel	Myrciinae	Uncertain	*Myrcia*	Not included in study	*Blepharocalyx-N. fernandeziana* group
Ugni candollei (Barnéoud) O. Berg	Myrtinae	Myrtinae	*Myrtus*	Not included in study	Not included in study
Ugni molinae Turcz.	Myrtinae	Myrtinae	*Myrtus*	*Myrteola* group	*Myrteola* group

Ugni selkirkii (Hook. and Arn.) O. Berg	Myrtinae	Myrtinae	*Myrtus*	Not included in study	Not grouped
Metrosideros stipularis (Hook. and Arn.) Hook.f.	Leptospermeae	Leptospermeae	*Metrosideros*	Outgroup	Not included in study

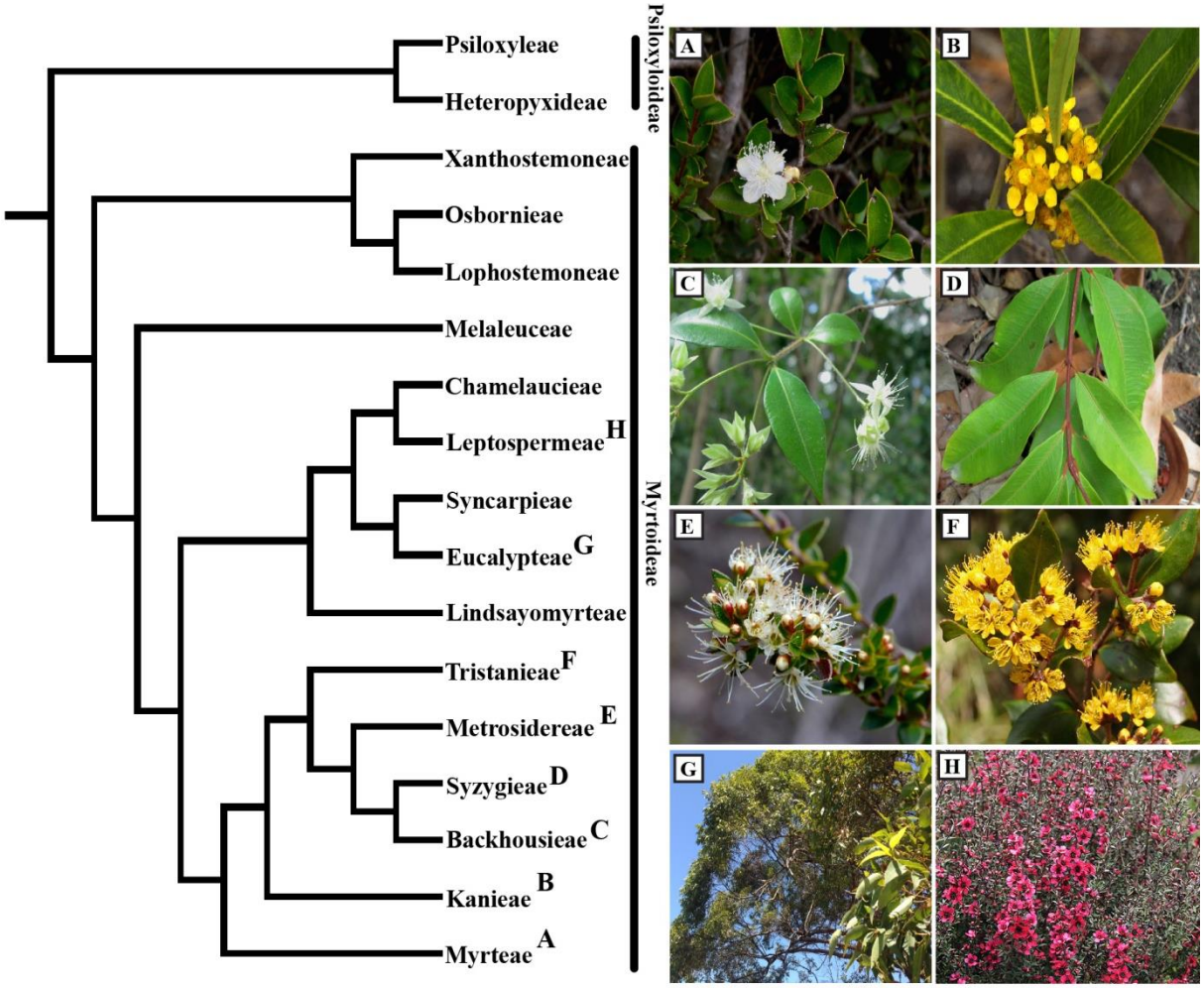


Figure 2.1. Cladogram of tribal phylogenetic relationships within Myrtaceae according to Wilson et al. (2005), Biffin et al. (2010) and Thornhill et al. (2015). (A-H) Representatives of each tribe used in this study as outgroups plus Myrteae: (A) *Luma apiculata* (tribe Myrteae). (B) *Tristaniopsis laurina* (tribe Kanieae). (C) *Backhousia myrtifolia* (tribe Backhousieae). (D) *Syzygium floribundum* (tribe Syzygieae). (E) *Metrosideros* (syn. *Tepualia*) *stipularis* (tribe Metrosidereae). (F) *Xanthomyrtus sp*. (tribe Tristanieae). (G) *Eucalyptus perriniana* (tribe Eucalypteae). (H) *Leptospermum scoparium* (tribe Leptospermeae).

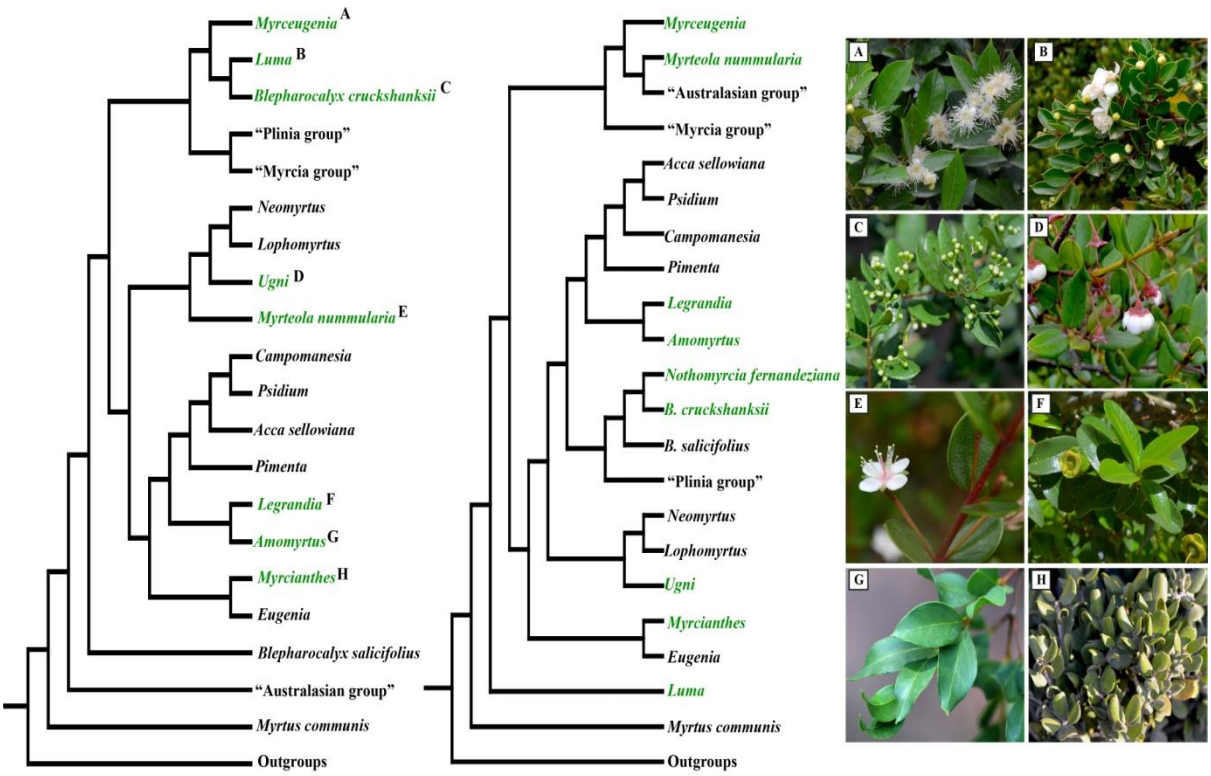
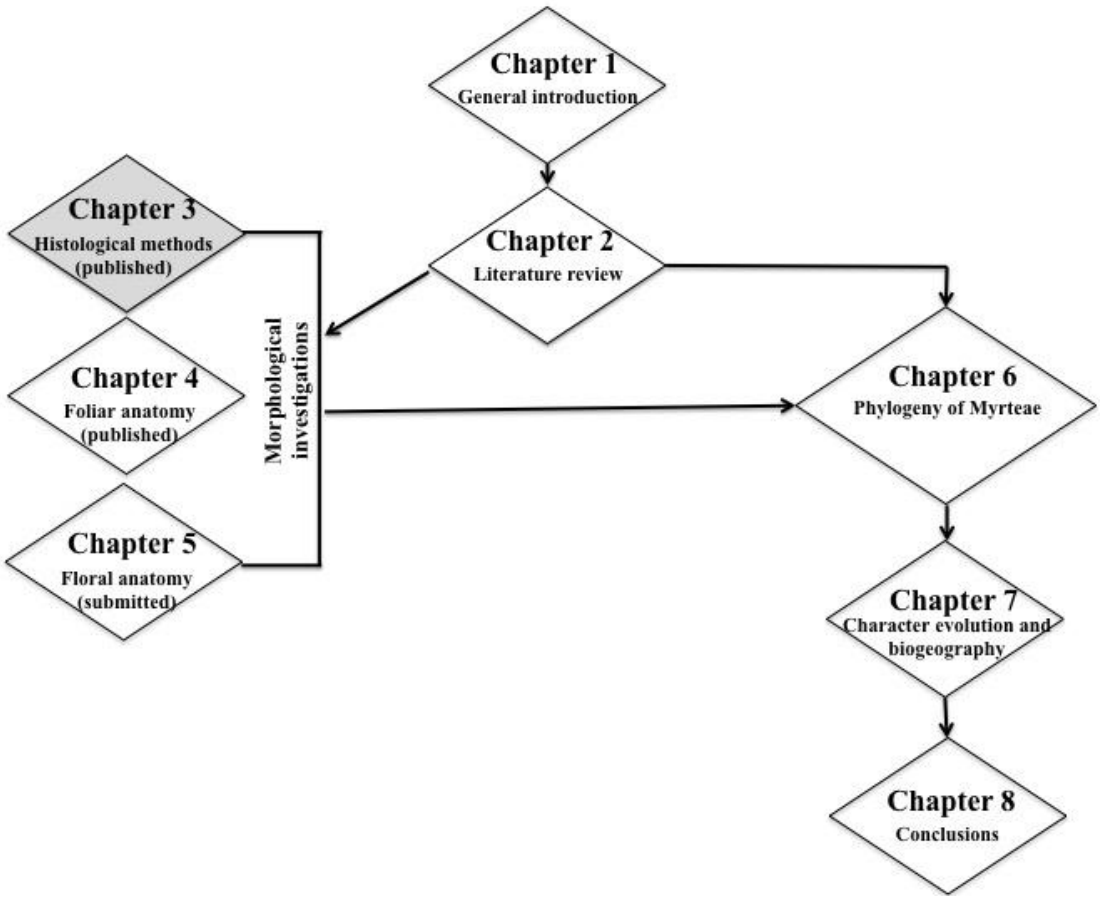


Figure 2.2. Comparison of phylogenetic relationships in Myrteae. Cladogram according to Lucas et al. (2007) (left) and Murillo et al. (2013, 2016) (right). Chilean taxa highlighted in green. (A-H) Representatives of Chilean Myrteae: (A) *Myrceugenia planipes*. (B) *Luma apiculata*. (C) *Blepharocalyx cruckshanksii*. (D) *Ugni candollei*. (E) *Myrteola nummularia*. (F) *Legrandia concinna*. (G) *Amomyrtus meli*. (H) *Myrcianthes coquimbensis*.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CHAPTER 3: A staining protocol for identifying secondary compounds in Myrtaceae

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Abstract

Here we propose a staining protocol using TBO and Ruthenium red in order to reliably identify secondary compounds in the leaves of some species of Myrtaceae. Leaves of 10 species representing 10 different genera of Myrtaceae were processed and stained using five different combinations of Ruthenium red and TBO. Optimal staining conditions were determined as 1 min of Ruthenium red (0.05% aqueous) and 45 sec of TBO (0.1% aqueous). Secondary compounds clearly identified under this treatment include mucilage in mesophyll, polyphenols in cuticle, lignin in fibres and xylem, tannins and carboxylated polysaccharides in epidermis and pectic substances in primary cell walls. Potential applications of this protocol include systematic, phytochemical and ecological investigations in Myrtaceae. It might be applicable to other plant families rich in secondary compounds and could be used as preliminary screening method for extraction of these elements.

Keywords

Anatomy; Myrtaceae; Ruthenium red; staining; Toluidine blue.

3.1 Introduction

Ammoniated ruthenium oxychloride (Ruthenium red) and the thiazine metachromatic stain Toluidine blue (TBO) are regarded as two effective biological stains (Chaffey et al., 2002). Both reagents are often used in plant staining protocols involving hydration, staining and dehydration (Johansen, 1940; Ruzin, 1999). TBO has been widely used in plant histology to highlight diverse anatomical components such as lignified and non-lignified cell walls, nuclei, polyphenols, tannins, suberin and others (O'Brien et al., 1964; Crews et al., 2003; Perez-de-Luque et al., 2006). TBO is a cationic stain that binds to tissue anions and gives two main spectra of reaction, purple-pink and green-blue (Baker, 1966). Ruthenium red is a polycationic stain that also has applications for electron microscopy (Luft, 1964; Colombo and Rascio, 1977). However, it also has important applications for light microscopy to stain aldehyde fixed mucopolysaccharides, calcium-dependent reactions and specific staining of pectic substances, mucilage and gums (Colombo and Rascio, 1977; Dierichs, 1979; Perez de-Luque et al., 2006). Improving the visual contrast of these reactions depends upon staining time, concentration and particular characteristics of the tissue, which might be species-dependent (Ruzin, 1999; Zhao et al., 2005). The visualization of these features can be

optimized using efficient staining protocols that stain certain chemical compounds contained for these structures (Johansen, 1940; Cutler et al., 2008).

In the case of the family Myrtaceae, compounds such as mucilage, pectins and polyphenols are abundant in the leaf mesophyll (Wilson, 2011). Mucilage, pectins and other chemical secretions are regarded as taxonomically significant characters for the family (Schmid, 1980; Keating, 1984; da Silva et al., 2012). Although Myrtaceae is a large family of plants with ca. 5500 species (Wilson, 2011), anatomical studies of leaves are scarce and reports on secondary compounds are limited. A number of species in the family are rich in chemical compounds with medicinal and biochemical activity (Wollenweber et al., 2000; Kytridis and Manetas, 2006). Nevertheless, pharmacological studies rely greatly in plant anatomy and more staining alternatives are needed in Myrtaceae.

Staining protocols used in Myrtaceae to date mainly involve Safranin O or some combination of Safranin O with Alcian blue, Astra blue or Fast green (Schmid, 1980; Gomes et al., 2009; Cardoso et al., 2009; Soh and Parnell, 2011). There are variety of studies regarding staining of plant tissues with Ruthenium red and TBO (Littlefield and Wilcoxson, 1962; Leiser, 1968; Western et al., 2001; Stpiczyńska and Davies, 2009). However, there are no published studies about optimization of staining procedures for a specific plant family or taxonomic group. Due to the presence of particular chemicals in the species of the family, an alternative staining protocol may improve the resolution of tissues in anatomical sections. Here we report an experiment using different combinations and duration of staining with Ruthenium red and TBO, so as to reliably identify secondary compounds in the family Myrtaceae.

3.2 Methods and results

3.2.1 Sampling, fixation and sectioning

Leaves of Australian and South American Myrtaceae from different genera were collected from the natural habitat of the species. Species were selected from different genera in order to encompass a diversity of leaf structures. Details of taxa, location and collector numbers are provided in Appendix 1. Voucher specimens are currently deposited at EIF with duplicates housed at BRI (Appendix 1). Leaves were fixed in FAA for 24-48h depending upon whether species had soft or hard leaves. Composition of FAA (for 100 ml) was 90 ml of 50% Ethanol, 5 ml of Glacial acetic acid and 5 ml of Formalin 37-40% (Johansen, 1940). Fixed material was dehydrated through a graded ethanol series and embedded in paraffin wax (Johansen,

1940; Ruzin, 1999). Transverse sections (5µm thickness) were cut using a Leica RM2245 rotary microtome.

3.2.2 Staining procedure

Samples were deparaffinised with xylene, and then gradually hydrated through a decreasing alcohol series (ethanol 100%, 90%, 70%, 50%, distilled water). Histochemical staining of sections was performed using a 0.1% (w/v) solution of Toluidine blue (TBO) (Amresco, Solon, Ohio, USA) in distilled water and 0.05% (w/v) of Ruthenium red (Sigma-Aldrich Co., Saint Louis, Missouri, USA) in distilled water following Jensen (1962). Samples were stained with one or both reagents for different periods of time according to five treatments, namely T1, T2, T3, T4 and T5 (Table 3.1). All specimens were subjected to these treatments (T1-T5) in order to determine optimal staining conditions that can be used to reliably identify anatomical characters across Myrtaceae. After staining, slides were dehydrated using an increasing ethanol series (50%, 70%, 90%, 100%, xylene) and mounted with DPX (Sigma-Aldrich Co., Saint Louis, Missouri, USA). The sections were observed using a Nikon SMZ 800 Stereo light microscope (Nikon eclipse 50i compound) and pictures were taken using the NIS Elements digital image analysis software (Nikon Instruments Inc., Amsterdam, Netherlands). Interpretation of colours from histochemical staining was based on O'Brien et al. (1964), Chaffei et al. (2002), Zhao et al. (2005) and Perez de-luque et al. (2006).

A total of 10 sections were stained per treatment for each species, which corresponds to ca. 500 sections. The entire staining experiment from deparaffinise to mounting, takes approximately two hours. Details of the staining protocol and cautionary comments are presented in the Appendix 4.

3.2.3 Optimal staining protocol

Histochemical reactions in leaves were notably different depending upon treatment. Staining with Ruthenium red for one minute and counterstaining with TBO for 45 sec (T5) proved to be the most effective combination for differentiating secondary compounds based on colour (Table 3.2). T5 also showed to be the most consistent treatment of the experiment, staining secondary compounds with similar colours and contrast uniformly in all the species. Under this treatment, polyphenols, carboxylated polysaccharides, mucilage and pectins were clearly visible in different parts of the leaf (Fig. 3.1). The treatment T5 allowed a proper contrast between the cuticle (blue-green for polyphenolic compounds) and the epidermal cells of most

of the species. Vascular bundles presented also better-defined elements using this treatment, showing clear differentiation between lignified secondary cell walls and non-lignified primary walls. Lignified vessels and fibres were stained blue-green with TBO, allowing excellent visual contrast. On the other hand, non-lignified primary cell walls in xylem, secondary phloem and non-vascular tissues were stained red with Ruthenium red, similarly to other studies in plants (Zhao et al., 2005; Perez de-luque et al., 2006). Positive staining with Ruthenium red was suitable for observing pectic substances in the middle lamella of non-lignified primary cell walls. Ruthenium red also allowed direct observation of mucilage in the mesophyll of most of the species.

Even though T5 had similar results through all the species in terms of secondary compounds, there are some taxa with special anatomical features stained differently. The palisade parenchyma cells of *Gossia floribunda* (A.J.Scott) N.Snow & Guymer, *Decaspermum humile* (Sweet ex G. Don) A.J. Scott and *Eugenia reinwardtiana* (Blume) DC.were stained strongly and appear darker than those of other species (Fig. 3.1) indicating the presence of tannins and polysaccharides. The epidermal cells of some species (*G. floribunda*, *E. reinwardtiana*, *Ugni molinae*) contain tannins (stained blue) and carboxylated polysaccharides (stained pink) whilst the epidermal cells of other species (*M. parvifolia, Syzygium australe* (J.C. Wendl. ex Link) B. Hyland, *Waterhousea floribunda* (F.Muell.) B.Hyland lack these compounds. The phloem sieve tube members of the midrib in certain taxa (such as *Acmena smithii* (Poir.) Merr. & L.M. Perry) have a dark-staining content, potentially tannins, whilst *M. parvifolia*, *U. molinae* and *W. floribunda* give contrasting examples of taxa without tannins in phloem.

The species *L. apiculata* and *M. parvifolia* reacted somewhat differently to the treatment T5, showing a different pattern and intensity of colours. Histochemical staining revealed abundance of pectic substances and mucilage in the mesophyll of *L. apiculata*, with a predominance of red staining over blue compared to the other species when treated with T5. In the case of *M. parvifolia*, staining was slightly weaker than the other nine species; however anatomical elements and secondary compounds were clearly differentiated.

3.2.4 Comparison between treatments

Treatment T1 showed similar results to T5 in terms of staining reaction, but some secondary compounds are not clearly visible with T1. Polyphenols (e.g. lignin) are stained red in the treatment T1, T2 and T4, without clear differentiation between the midrib fibres and the mesophyll cells due to weak reaction of TBO (Fig. 3.2). Similarly, xylem and phloem are not

easily differentiable under T2, T3 and T4 (Fig. 3.3). On the other hand, T5 allowed the clear observation of lignin in fibres stained blue for this dye (Fig.3.2) and polyphenols in cuticle (Fig. 3.3). Although there are some differences if the lignin is fresh (*in situ*), polychromatic staining with TBO is a reliable method to identify this compound when the staining time is optimal (O'Brien et al., 1964). Overstaining with Ruthenium red (T2, T4) produced homogeneous red staining through the samples without any coloured enhancement of secondary compounds excepting for mucilage and pectic substances (Fig. 3.2, Fig. 3.3). In the case of T4, the action of TBO might be neutralized by Ruthenium red, which is regarded as a stronger stain (Dierichs, 1979; Chaffei et al., 2002). Treatment T3 with TBO for 2 minutes resulted in blue overstaining without the optimal polychromatic reaction in tissues.

The combination that showed the best results (T5) might be proposed as an alternative protocol to existing ones involving different stains in Myrtaceae. Procedures involving Safranin O or combination of Safranin O with Alcian blue, Astra blue or Fast green have shown satisfactory results in anatomical studies on the family. However, there are no published studies supporting the use of these stains and their advantages. In addition to the quality of the enhancement of secondary compounds, the use of Ruthenium red and TBO has advantages in terms of time and safety. Safranin O is a regressive stain and needs between 2 and 24 hours to be effective and requires destaining in distilled water (Johansen, 1940; Ruzin, 1999). Safranin O also requires differentiation with picric acid, hydrochloric acid or tannic acid, regarded as unstable reagents (Ashbrook et al., 2003). On the other hand, the use of Ruthenium red and TBO does not require much time (2-3 minutes) or dangerous reagents.

3.3 Conclusion

In this paper we introduced a double staining protocol using Ruthenium red and TBO. We have evaluated a number of different staining treatments with these reagents in order to reliably differentiate secondary compounds in leaves of some species of Myrtaceae. The best combination was determined as one minute of Ruthenium red and 45 seconds of TBO (T5). Under this treatment, a number of secondary leaf compounds can be clearly identified: polyphenols, mucilage, carboxylated polysaccharides and pectic substances. This procedure enhances the contrast of secondary compounds, which are visible in a wide range of colours (green-blue-red-pink). The applicability, safety and effectiveness are the main advantages of this protocol when compared to similar staining procedures used in the family. Other staining protocols used in Myrtaceae require more time and involve unstable reagents as hydrochloric

acid, tannic acid or even explosive compounds such as picric acid. This protocol involves relatively few reagents and offers the option of adjusting and varying the duration at each stage of the process. This procedure might be alternative to commonly used staining protocols in Myrtaceae. Although the best results were obtained using a certain combination of stains, it is advisable to test the full procedure in order to detect differences in the results with other taxonomic groups. Identification of secondary compounds in leaves of Myrtaceae is highly important for systematic, phytochemical and ecological studies. This protocol could be used as a screening method for deeper study or extraction of these compounds.

Table 3.1. Staining treatments applied in this study based on duration of staining.

	Duration of Staining (min)	
Treatment	Ruthenium red (0.05% w/v)	TBO (0.1% w/v)
T1	2	1
T2	2	0.5
T3	0	2
T4	2	0
T5	1	0.75

Table 3.2. Secondary compounds identified with each treatment for all the species based on the colour resulting from the staining process.

	Secondary compounds (colours)																			
	Mucilage (red-pink)					Polyphenols, tannins, lignin (blue-green)					Carboxylated polysaccharides (pink)					Pectic substances and some tannins (red)				
Taxa / Treatments	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Acmena smithii	+	+	-	+	+	+	+	-	-	+	+	-	-	-	+	-	+	-	+	+
Gossia floribunda	+	+	-	+	+	+	-	-	-	+	+	+	-	+	+	+	+	-	+	+
Decaspermum humile	-	+	-	+	+	-	-	-	-	+	+	+	-	+	+	+	+	-	+	+
Eugenia reinwardtiana	-	+	-	+	+	-	-	-	-	+	+	+	-	+	+	-	+	-	+	+
Luma apiculata	+	+	-	+	+	+	-	-	-	+	+	-	-	-	+	+	+	-	+	+
Myrceugenia parvifolia	-	+	-	+	+	+	-	-	-	+	+	+	-	-	+	-	+	-	+	+
Myrteola nummularia	-	+	-	+	+	+	-	-	-	+	+	+	-	-	+	-	+	-	+	+
Syzygium australe	-	+	-	+	+	+	+	-	-	+	+	+	-	-	+	-	+	-	+	+
Ugni molinae	+	+	-	+	+	+	+	-	-	+	+	-	-	+	+	-	+	-	+	+
Waterhousea (Syzygium) floribunda	+	+	-	+	+	+	+	-	-	+	+	-	-	-	+	-	+	-	+	+

(+) Positive staining of secondary compounds
(-) No staining of secondary compounds

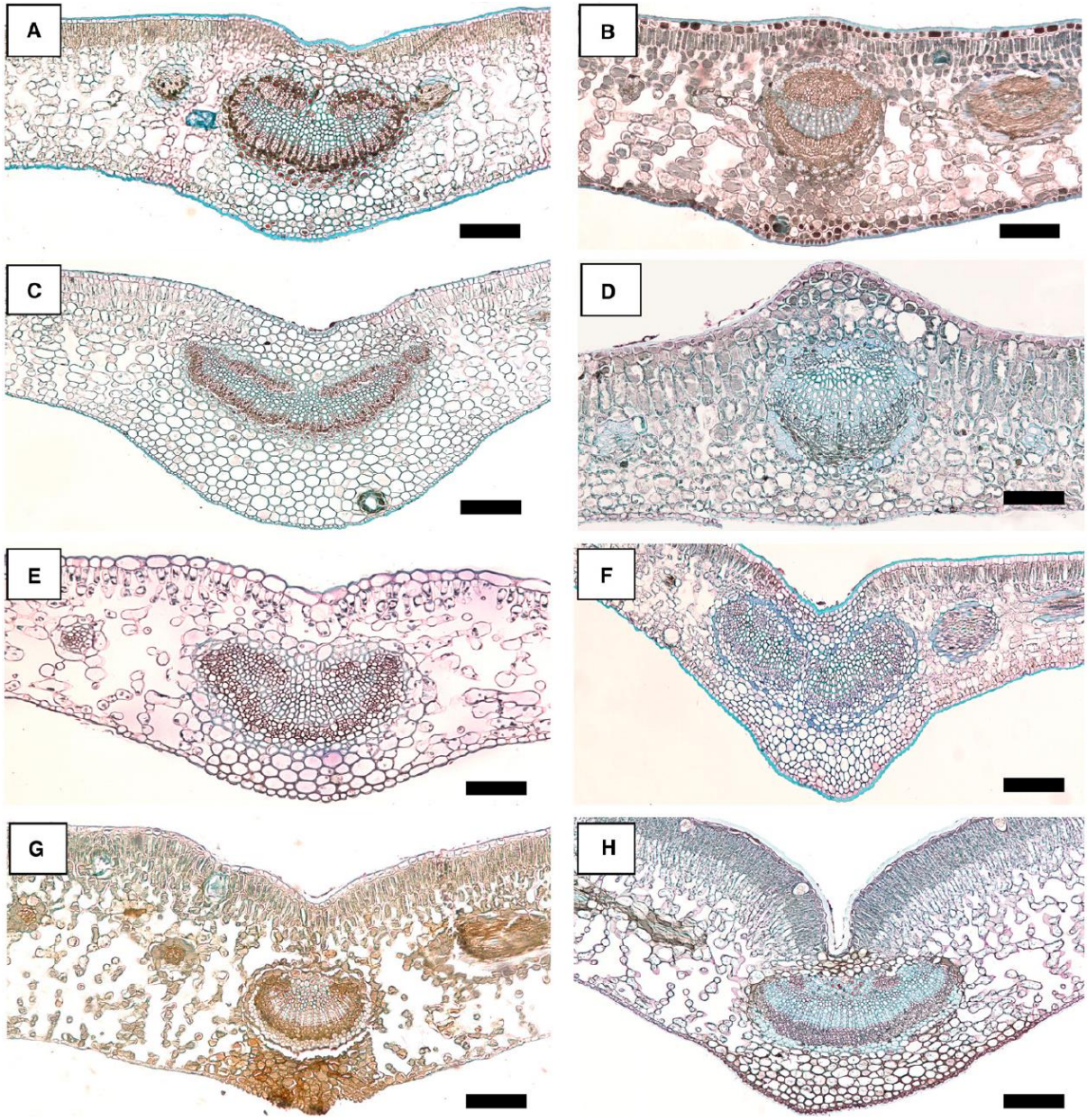


Figure 3.1. Transverse light micrographs (LM) of leaves of some species of Myrtaceae stained with the staining treatment T5. A, *Acmena smithii*: Clear highlight of lignified cells (blue) and mucilage (red). B, *Eugenia reinwardtiana*: Carboxylated polysaccharides in epidermis. C, *Syzygium australe*: Phloem cells stained red and xylem blue-green. D, *Gossia floribunda*: Mesophyll cells containing dark stained pigments, probably tannins. E, *Myrceugenia parvifolia*: Pectic substances in primary cell walls observed in spongy parenchyma (red). F, *Waterhousea floribunda*: Cuticle with polyphenols stained blue and fibres with lignin stained blue-purple. G, *Luma apiculata*: Red staining of pectic substances and mucilage. H, *Ugni molinae*: Mucilage in spongy parenchyma and clear difference between polyphenols in xylem (lignin) and phloem. Scale bars = 100 µm.

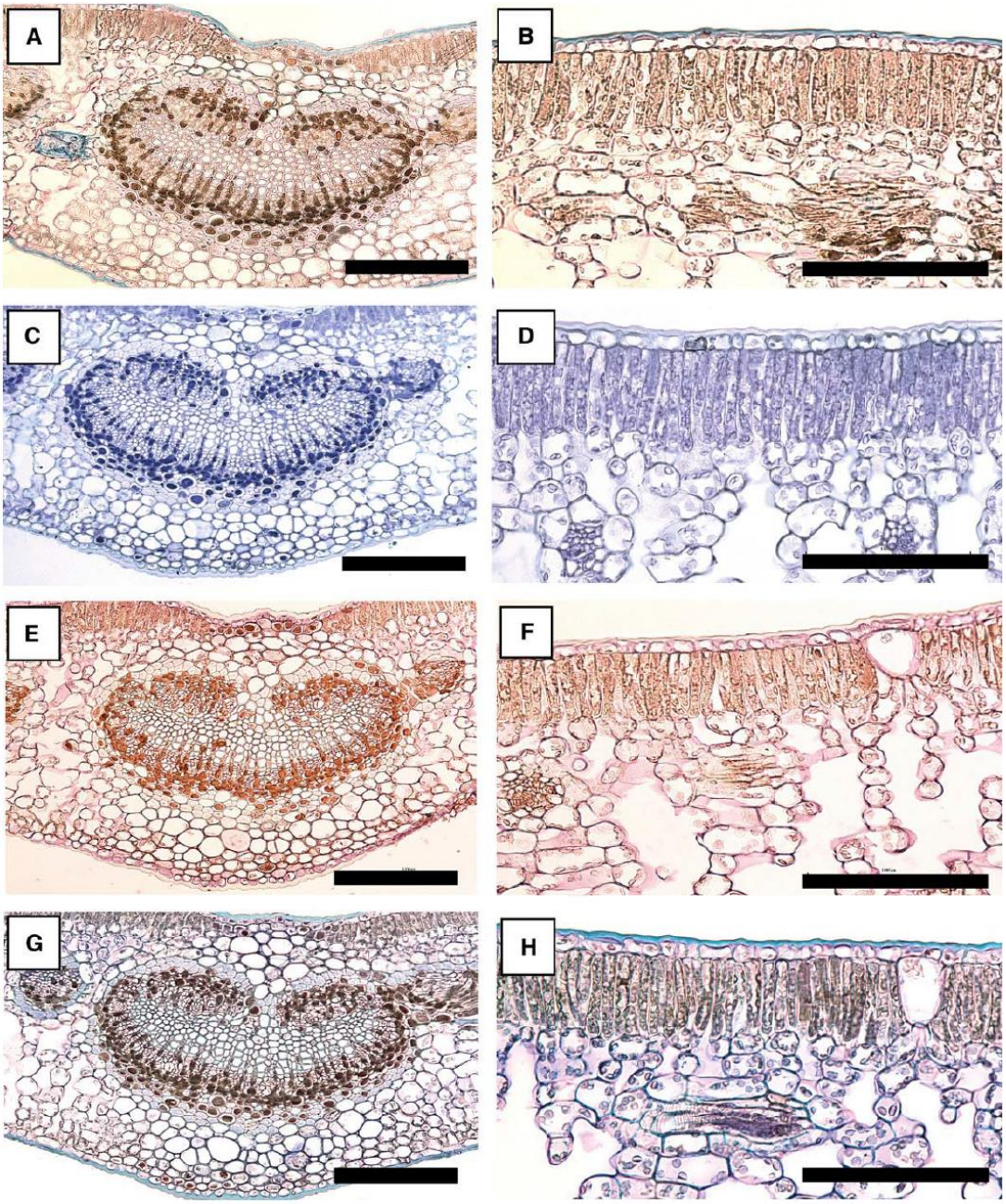


Figure 3.2. Transverse light micrographs (LM) of leaves of *Acmena smithii*, showing comparisons between treatments T2 (A-B), T3 (C-D), T4 (E-F) and T5 (G-H). Treatment T1 showed similar results to T5. A-B, Polyphenols highlighted in cuticle (blue) and mucilage (red), however lignified elements in midrib are not differentiated. C-D, Secondary compounds are not differentiated for masking of TBO. E-F, Secondary compounds are not differentiated for masking of Ruthenium red. G-H, Secondary compounds clearly visible in T5. Polyphenols in cuticle, xylem and fibers (lignin, blue), mucilage and pectic substances in mesophyll (red), tannins in phloem (red) and carboxylated polysaccharides in epidermis (pink). Scale bars = 100 µm.

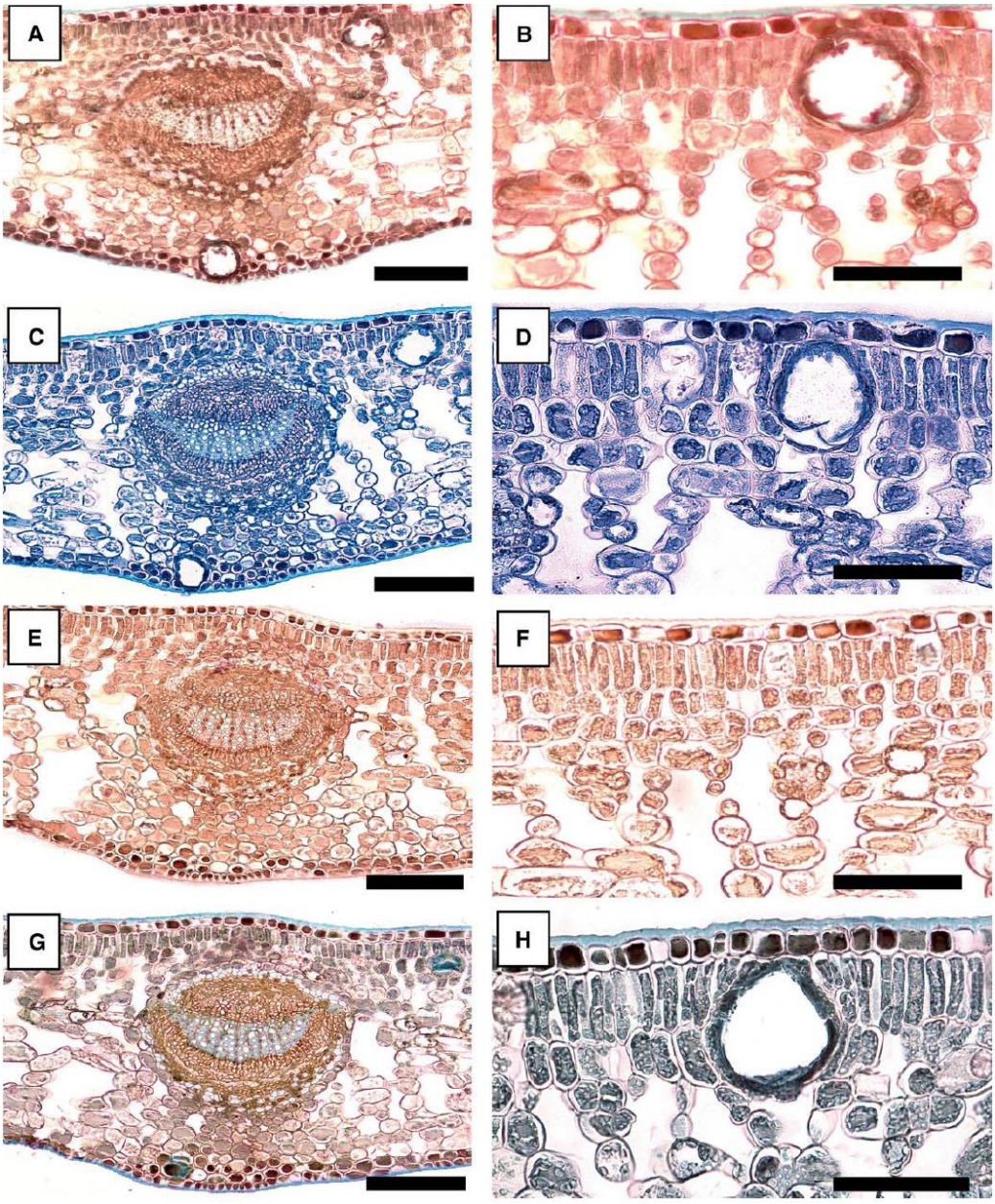
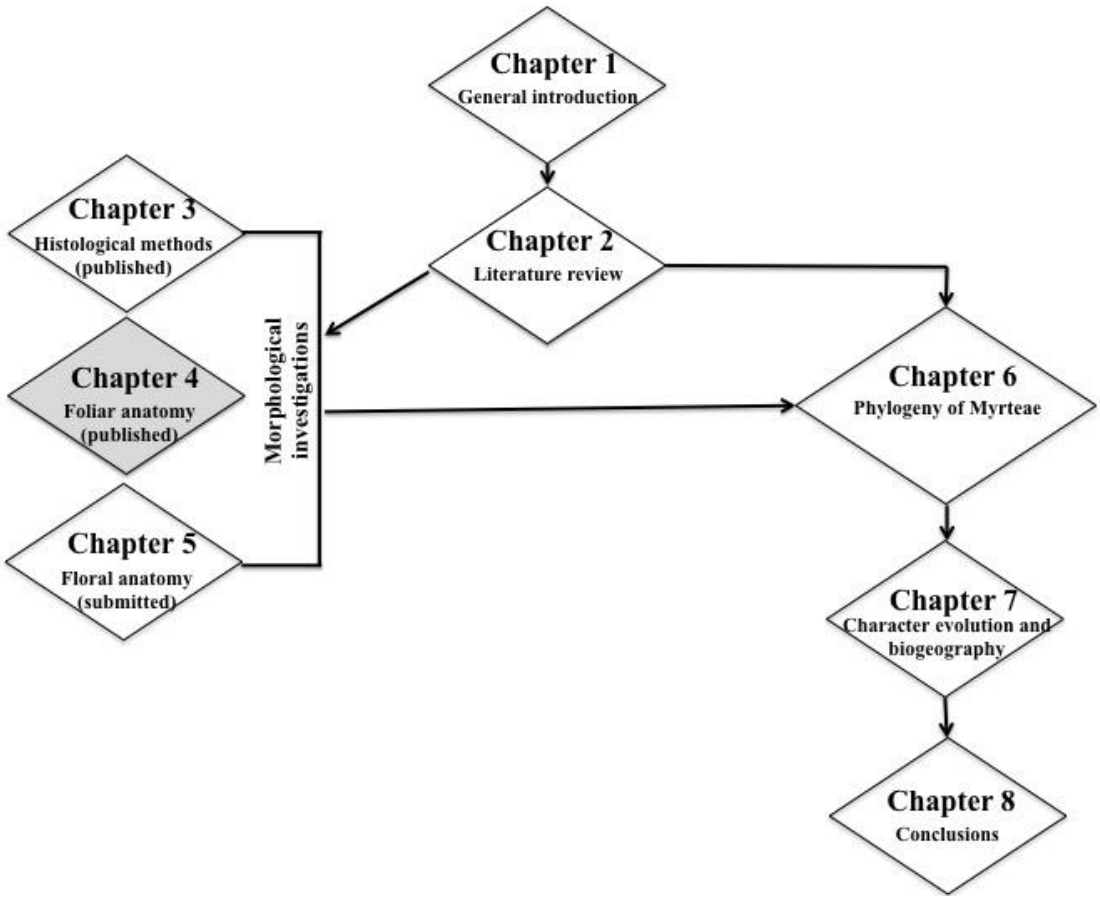


Figure 3.3. Transverse light micrographs (LM) of leaves of *Eugenia reinwardtiana*, showing comparisons between treatments T2 (A-B), T3 (C-D), T4 (E-F) and T5 (G-H). Treatment T1 showed similar results to T5. A-B, Only polyphenols in cuticle (blue) are differentiated as the red staining is predominant. C-D, Secondary compounds are not differentiated for masking of TBO, except for the cuticle. E-F, Secondary compounds are not differentiated for masking of Ruthenium red. G-H, Secondary compounds clearly visible in T5. Clear difference between xylem and phloem. Polyphenols in cuticle, xylem and fibers (lignin, blue), mucilage and pectic substances in mesophyll (red), tannins in phloem (red) and carboxylated polysaccharides in epidermis (pink). Red tannins in epidermis observable with all the treatments. Scale bars = 100 µm.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CHAPTER 4: Comparative leaf anatomy and micromorphology of the Chilean Myrtaceae: Taxonomic and ecological implications

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Abstract

The family Myrtaceae in Chile comprises 26 species in 10 genera. The species occur in a diverse range of environments including humid temperate forests, swamps, riparian habitats and coastal xeromorphic shrublands. Most of these species are either endemic to Chile or endemic to the humid temperate forests of Chile and Argentina. Although many taxa have very restricted distributions and are of conservation concern, little is known about their biology and vegetative anatomy. In this investigation, we describe and compare the leaf anatomy and micromorphology of all Chilean Myrtaceae using standard protocols for light and scanning electron microscopy. Leaf characters described here are related to epidermis, cuticle, papillae, stomata, hairs, mesophyll, crystals, secretory cavities and vascular system. Nearly all the species have a typical mesophytic leaf anatomy, but some species possess xerophytic characters such as double epidermis, hypodermis, pubescent leaves, thick adaxial epidermis and straight epidermal anticlinal walls, which correlate with the ecological distribution of the species. This is the first report of leaf anatomy and micromorphology in most of these species. We identified several leaf characters with potential taxonomic and ecological significance. Some combinations of leaf characters can reliably delimitate genera, while others are unique to some species. An identification key using micromorphological and anatomical characters to distinguish genera and species is provided.

Keywords: identification key - histochemistry- taxonomy - Valdivian forest - xeromorphic

4.1 Introduction

Myrtaceae Juss. (Myrtales; APG IV, 2016) is a large family of angiosperms with approximately 5500 species, divided into two subfamilies, 17 tribes and ca. 140 genera (Biffin et al., 2010; Wilson, 2011). It is a predominantly southern hemisphere family with a high diversity in South America and Australasia (Snow, 2000). In Chile, the family is represented by 26 species in 10 genera distributed from the north-centre to the southern tip of the mainland region and in the Juan Fernandez Islands (Landrum, 1988a; Murillo and Ruiz, 2011). All Chilean species of Myrtaceae belong to the tribe Myrteae, with the exception of *Metrosideros stipularis* (Hook. and Arn.) Hook f., which is in the tribe Metrosidereae (*sensu* Wilson et al., 2005).

Most of the Chilean Myrtaceae occur in the "Chilean Winter Rainfall-Valdivian Forest Hotspot", a biogeographic area located in between 25º and 47º south latitude. This region is

widely known for having a high level of plant endemism (Arroyo et al., 2004). Part of this biogeographic region is considered priority for plant conservation at global scale (Myers et al., 2000). This area includes the Juan Fernandez Islands, where three species of Myrtaceae are endemic, namely *Myrceugenia schulzei*, *Nothomyrcia fernandeziana* and *Ugni selkirkii* (Landrum, 1988a). Most species of Chilean Myrtaceae occur in humid temperate forests or flooded environments, usually wet gullies or streams (Kausel, 1942, 1956). The Chilean Myrtaceae are an abundant component in the upper, middle and even lower strata of these forests (Hildebrand-Vogel, 2002). A few species, such as *Myrceugenia rufa* (Colla) Skottsb. ex Kausel and *Myrcianthes coquimbensis* (Barnéoud) Landrum & Grifo, occur exclusively in dry habitats with the water supply limited to fog and ocean breeze (Serra et al., 1986; Landrum and Grifo, 1988). *Myrceugenia correifolia* occurs in coastal xeromorphic habitats in central Chile, with some populations in cloud forests (Landrum, 1981b).

Leaf anatomical characters have provided valuable systematic and ecological information in Myrtaceae. Metcalfe and Chalk (1979), Schmid (1980) and Keating (1984) described leaf anatomical characters at family level with important taxonomic implications. Cardoso et al. (2009) and Gomes et al. (2009) conducted detailed leaf anatomical studies in several South American species, indicating that anatomical characters, alongside morphological features, can be used to identify species and genera. Based on leaf anatomical characters and DNA sequences, Soh and Parnell (2011) reconstructed the phylogeny of the Australasian genus *Syzygium* and found a number of characters useful in delimiting sections and species. Leaf micromorphology (using SEM) of South American Myrtaceae has been mainly studied in *Eugenia* and shown to be important for taxonomic purposes (Fontenelle et al., 1994; Haron and Moore, 1996).

The leaf anatomy and micromorphology of the Chilean Myrtaceae has not been documented in much detail (P.G. Wilson, pers. comm.), other than a few species, namely *Luma apiculata*, *Myrceugenia parvifolia* and *Ugni molinae* (Retamales and Scharaschkin, 2014). There has never been a comprehensive study of the Chilean Myrtaceae other than taxonomic revisions based on gross morphological characters (Kausel, 1942; Landrum, 1981b, 1986, 1988a; McVaugh, 1968; Reiche, 1897). The Chilean Myrtaceae show high variation in gross morphology of leaves between species (Fig. 4.1) and also within same species, which precludes diagnosis and species identification (McVaugh, 1968). A complete anatomical investigation of these taxa could provide relevant information by identifying reliable characters with taxonomic and ecologic significance. In this investigation, we present the

outcome of extensive research on the anatomical and micromorphological characters of leaves of all the species of Myrtaceae occurring in Chile.

4.2 Material and methods

4.2.1 Material examined

All 26 species of Chilean Myrtaceae were examined in this study. Wherever possible, fresh leaf material was collected but in a few cases herbarium specimens (CONC) were used. Sampling was conducted between January 2006 and February 2014 and included a number of different natural populations in Chile. Mature leaves were randomly sampled from sun-exposed branches from a number of typical and healthy individuals. Young leaves were also collected as trichomes and certain other structures are reported to be early caducous (Landrum, 1988a). Young leaves were also used to describe early ontogenetic stages of secretory cavities and epidermis. Fresh leaf material was fixed in formalin-acetic acid-alcohol (FAA) for 24-48 h depending upon the thickness of the leaves and subsequently stored in 70% ethanol. Herbarium specimens were rehydrated in boiling water for 10 min to recover the leaf shape before being fixed in FAA (Haron and Moore, 1996). Herbarium accessions are currently deposited in the Forestry Sciences Herbarium, University of Chile (EIF), while duplicates are housed in the Queensland Herbarium, Brisbane, Australia (BRI). Details of specimens studied, vouchers, localities and habitat are presented in the Appendix 1.

4.2.2 Scanning electron microscopy (SEM)

Leaf material fixed in FAA was dehydrated using a graded ethanol series and then critical point dried (Anderson, 1951) in an Autosamdri-815 automatic critical point drier (Tousimis, Rockville, USA). Samples were mounted on stubs with self-adhesive double-sided carbon discs and sputter-coated with gold palladium for 175 sec using a Leica EM SCD005 Gold Coater (Leica Microsystems, Macquarie Park, NSW, Australia). Examination and documentation of images was conducted using a FEI Quanta 200 SEM/ESEM (FEI, Hillsboro, Oregon, USA) operated at 10kV.

4.2.3 Light microscopy (LM)

FAA-fixed material was dehydrated through a graded ethanol series and embedded in paraffin wax (Johansen, 1940; Ruzin, 1999). Transverse sections of leaves were cut using a Leica RM2245 rotary microtome (Leica Microsystems, Macquarie Park, NSW, Australia) at

5µm. Staining of sections was performed using the stains ruthenium red (0.05% aqueous solution), toluidine blue (TBO) (0.1% aqueous solution), safranin O (1% alcoholic solution) and alcian blue, alone or combined according to standard staining protocols (Ruzin, 1999; Retamales and Scharaschkin, 2014). In order to reliably identify the chemical compounds in tissues, additional histochemical tests were performed in unstained leaves using the reagents sudan IV, chlorazol black E and phloroglucinol (20% HCl) to detect lipophilic substances and lignin. Chemical nature of leaf intracellular crystals was tested by adding 1µl of acetic acid and 1µl of hydrochloric acid to sections (Maclean and Ivimey-Cook, 1952). Sections were mounted using DPX (Sigma-Aldrich Co., St. Louis, Missouri, USA).

Leaf clearings were prepared by immersing 1-2 cm^2 pieces of leaf material in 10% KOH at room temperature for 48 h followed by 7% NaClO for 2 h or until leaves turned transparent (Gardner, 1975). Cleared leaves were washed five times with distilled water, stained with 1% safranin O and mounted with lactoglycerol (lactic acid-glycerol 1:1). Slides were observed using a Nikon eclipse 50i compound microscope and images captured using the Nikon NIS-Elements imaging software (Nikon Instruments Inc., Amsterdam, Netherlands).

4.2.4 Taxonomy and terminology

The taxonomy of Chilean Myrtaceae is based on Landrum (1988a) and follows the author abbreviations of The Plant List (2016), with two exceptions. *Myrceugenia fernandeziana* (Hook. and Arn.) Johow is considered here as *Nothomyrcia fernandeziana* (Hook. and Arn.) Kausel based on Murillo and Ruiz (2011) and *Tepualia stipularis* is considered as *Metrosideros stipularis*, following Pillon et al. (2015) and WCSP (2016). The abbreviation spp. will be used for referring to all species included in this study from a particular genus. In order to avoid ambiguities, the genera with the root *Myr–* (*Myrceugenia*, *Myrcianthes*, *Myrteola*) will not be abbreviated in the text other than in the anatomical synopsis of genera. Taxonomic authorities of species are shown in Appendix 1; therefore these have been omitted from the text henceforth.

The five types of stomatal complexes studied here were anomocytic, paracytic, actinocytic, anisocytic and laterocytic. When more than one type of stomatal complex was identified in some species, the less frequent type is indicated in parentheses (Table 4.1). The description and interpretation of the different stomatal types in Chilean Myrtaceae are as follows: (1) Anomocytic: the guard cells are surrounded by unspecialized subsidiary cells without any consistent pattern and that are indistinguishable in shape from other epidermal cells. (2)

Paracytic: the guard cells are surrounded by two subsidiary cells, which are relatively specialized. These two cells are normally parallel with the long axis of the guard cells and are generally similar in size. (3) Actinocytic: the guard cells are surrounded by four or more, usually radially elongated, subsidiary cells. (4) Anisocytic: the guard cells are surrounded by three cells that are usually unequal in size. One of the three cells is usually much smaller than the other two. (5) Laterocytic: the guard cells are surrounded by six irregularly shaped subsidiary cells.

In order to reliably identify different types of secretory cavities, we observed ontogenetic stages in young leaves. Secretory cavities initially formed by dissolution of cells are classified as lysigenous, while those formed by initial separation of epithelial cells are classified either as schizogenous or schizolysigenous (Ciccarelli et al., 2008). Multiple layers of epidermis are classified as hypodermis when the additional layer(s) of large epidermis-like cells are derived from the ground meristem or as multiple epidermis, when these cells originate from the protodermis. (Martins et al., 2012)

Terminology for describing leaf micromorphology (mainly stomata) was based on previous descriptions of van Wyk et al. (1982), Fontenelle et al. (1994), Haron and Moore (1996) and Soh and Parnell (2011). Terminology for leaf anatomy was based on Schmid (1980), Schmid and Baas (1984), Keating (1984), Cardoso et al. (2009), Soh and Parnell (2011) and Retamales et al. (2015). Other general references consulted for anatomical terminology were Gifford and Foster (1989), Dickison (2000), Evert (2006) and Pole (2010).

4.3 Results

The results will be presented in three parts: (1) A survey of the leaf anatomical and micromorphological characters, (2) a synopsis of the leaf anatomy of each genus and (3) an identification key of Chilean species of Myrtaceae using anatomical and micromorphological characters. Leaf characters are summarized in Table 4.1 and Table 4.2.

4.3.1 Survey of leaf characters

Epidermis, cuticle and epicuticular waxes

Different types of anticlinal walls of abaxial epidermal cells are observed. The most common type is the slightly sinuous with thin walls, present in the majority of taxa. Some species possess sinuous cell walls (Figs 4.2A, 4.2B, 4.2C) while others have straight and thick walls

(Fig. 4.2D). Adaxial epidermal cells have straight or straight-sinuous anticlinal walls in all cases.

The epidermal cell walls are mucilaginous (evidenced by test with ruthenium red), single layered in most of the species (Fig. 4.3A) and generally thicker on the adaxial side of the leaf. The species *Myrceugenia correifolia*, *Myrceugenia obtusa* and *Myrcianthes coquimbensis* possess a very thick adaxial epidermis, sometimes with a diffuse second layer beneath. Adaxial epidermal cells have thin primary cell walls and are plano-convex and mainly isodiametric in shape in the majority of taxa. Some species have enlarged-rectangular epidermal cells. Both species of *Luma* have isodiametric and enlarged-rectangular epidermal cells distributed equally on the adaxial surface. *Myrceugenia colchaguensis* possesses irregularly shaped epidermal cells. The pattern of the epidermal cells (shape and size) changes above the main vascular bundle in *Amomyrtus* spp. and *Legrandia concinna* but remains unchanged in the majority of the species. The species *N. fernandeziana*, *U. candollei* and *Myrceugenia rufa* possess extra subepidermal cell layers. Observations in young leaves showed that the subepidermal layer in *N. fernandeziana* and *U. candollei* possibly correspond to hypodermis as this tissue is related to ground meristem in origin (Fig. 4.3B). On the other hand, the homogenous subepidermal layer observed in *Myrceugenia rufa* is originated from the protodermis, which suggests that the species has a multiple (double) epidermis (Fig. 4.3C). Abaxial epidermal cells are small, rounded and isodiametric in nearly all the species. *Myrceugenia obtusa* and *Myrcianthes coquimbensis* have larger abaxial epidermal cells, with nearly 1:1 relative size to adaxial epidermal cells. Conical papillae can be observed on both adaxial and abaxial surfaces in some species. When present, papillae are combined with cuticular striations.

The cuticle is thicker on the adaxial surface than the abaxial surface in all species. The cuticular layer is either thin (3µm or less) in a majority of the species, but in some (such as *Myrceugenia correifolia*, *Myrceugenia rufa* and *Myrcianthes coquimbensis*) it is thick (>5 µm, up to 8 µm). The cuticle has ornamentations of epicuticular waxes in some taxa (Fig. 4.3D). Epicuticular waxes, as observed by SEM are granules or flakes. *Myrceugenia lanceolata* has very abundant epicuticular waxes on the abaxial surface, which gives a whitish colour to this side of the leaves.

Stomata

All species have hypostomatic leaves, except for *Myrteola nummularia*, which has amphistomatic leaves (stomata on both adaxial and abaxial surfaces). Stomata protrude slightly above the level of the epidermis (Figs 4.4G, 4.4H, 4.4I). Anomocytic stomata were observed in *Amomyrtus spp.*, *B. cruckshanksii*, *L. concinna*, most of the *Myrceugenia* species, *U. molinae* and *U. selkirkii* (Fig. 4.2E). Paracytic stomata were observed in in *L. apiculata* and *L. chequen* (Fig. 4.2F). Actinocytic stomata are common in *Myrceugenia colchaguensis.* Anisocytic stomata are the most common type in *U. candollei* (Fig. 4.2G). Laterocytic stomata are common in *Myrcianthes coquimbensis* (Fig. 4.2H). In transverse section, differences in the shape of guard cells and the degree of cutinized thickenings on the outer periclinal cell walls of guard cells can be observed. Guard cells are triangular and have cutinized thickenings of outer periclinal walls in some species (*Myrceugenia lanceolata* and *Myrceugenia planipes* (Fig. 4.3E)). Ovate guard cells without cutinized thickenings were observed in *L. chequen* (Fig. 4.3F), while *L. apiculata* shows ovate guard cells with heavy cutinized thickenings (Fig. 4.3G). Irregular thickenings were observed in *U. selkirkii* (Fig. 4.3H).

Indumentum

The majority of the species have sparsely pubescent leaves on both adaxial and abaxial surfaces (Fig. 4.5A). The leaves in most of the species become glabrescent with age. Only two species (*M. stipularis* and *B. cruckshanksii)* have completely glabrous leaves, where hairs were not observed in either young or mature leaves. Four species, namely *U. candollei* (Fig. 4.5F), *Myrceugenia correifolia* (Fig. 4.5B), *Myrceugenia exsucca* and *Myrceugenia planipes*, have sparse to moderately pubescent indument, particularly on the abaxial surface. Abaxially lanate (densely hairy) leaves were observed in *Myrceugenia colchaguensis*, *Myrceugenia rufa* (Fig. 4.5C) and *Myrceugenia schulzei* (Fig. 4.5E). Abaxially and adaxially lanate leaves were observed in *Myrcianthes coquimbensis* (Fig. 4.5D).

Three types of unicellular hairs were observed: simple (straight, curved, hooked, twisted or ciliate) (Figs 4.6A, 4.6C), dibrachiate (symmetrically or asymmetrically dibrachiated) (Fig. 4.6B) and glandular (Figs 4.6D, 4.6E, 4.6F). Simple hairs are observed in *L. concinna*, *Myrcianthes coquimbensis*, *Myrteola nummularia*, *N. fernandeziana, U. molinae* and *U. selkirkii*. Dibrachiate hairs were observed in *Myrceugenia spp.*, with some species also possessing simple hairs. Hairs are appressed in some species, especially in the case of

dibrachiate hairs. Glandular hairs were observed in *L. chequen*, *Myrceugenia colchaguensis*, *Myrceugenia obtusa* and *Myrcianthes coquimbensis.* A distinctive staining reaction to TBO is detected in some glandular hairs of *M. obtusa,* which probably indicated the presence of sesquiterpenes.

Mesophyll

All taxa have dorsiventral mesophyll with palisade parenchyma composed of rectangular, attenuated and vertical cells. The number of cell layers of the palisade parenchyma varies from a single layer in *Myrceugenia parvifolia* (Fig. 4.7A) to three distinct layers in *Myrceugenia lanceolata* (Fig. 4.7B) and *L. concinna* (Fig. 4.7C). Four compressed layers were observed in *Myrceugenia rufa* (Fig. 4.7D). The remaining taxa have two layers of palisade parenchyma, usually with a diffuse and poorly developed third layer (Fig. 4.7E). The spongy parenchyma is composed of irregularly shaped cells that vary from rounded to polygonal. Intercellular spaces do not vary considerably between taxa. The staining reaction to ruthenium red confirms the presence of mucilage and pectins in the mesophyll of all the species. The mesophyll of *Myrcianthes coquimbensis* (Fig. 4.7F) and *U. selkirkii* is rich in tannins and polyphenols. *Legrandia concinna* possesses domatia covered with ciliate hairs on the abaxial side of leaves, which are originated from the mesophyll. Domatia are easily observed in the axils of the midrib and the secondary veins of *L. concinna* (Fig. 4.4D).

Crystals

Intracellular crystals are present in most of the species. Two main types of crystals were found, namely druses (aggregated individual crystals) and prismatic crystals (rhombohedral and spherical). The chemical composition of crystals was determined by testing with acetic acid and hydrochloric acid. If the crystals dissolved, the composition was inferred to be CaOx (calcium oxalate) (Maclean and Ivimey-Cook, 1952). Druses are mainly contained in idioblasts and present in the palisade parenchyma below the adaxial epidermis (Fig. 4.3J). In some species, druses are also present around the leaf phloem and contained in bundle sheath cells. *Myrceugenia colchaguensis, Myrceugenia schulzei* and *U. selkirkii* exhibit prismatic rhombohedral crystals, mainly around the vascular system (Fig. 4.3K). Two species (*Myrceugenia chrysocarpa* and *Myrceugenia planipes*) possess spherical crystals located below the epidermis and also throughout the spongy parenchyma (Fig. 4.3I). Idioblasts with druses are mainly solitary or occur in pairs, however in some species (e.g., *L. concinna*)

several idioblasts are grouped together (Fig. 4.3L). Druses were not observed in the leaves of *M. stipulari*s and appear to be rare in *Myrcianthes coquimbensis.*

Secretory cavities

Leaf secretory cavities are generally located in the palisade parenchyma, usually in contact with the adaxial epidermis (Fig. 4.3O) but in some species they are located below both adaxial and abaxial surfaces. In young leaves, all cavities are initially formed by separation of epithelial cells (Fig. 4.3M), which confirms that secretory cavities in Chilean Myrtaceae are not lysigenous (cavities formed by dissolution of cells). Species have either schizogenous or schizolysigenous cavities (a mixture of schizogenous and lysigenous cavities). In early developmental stages, epithelial cells of schizogenous cavities are small, isodiametric and have very thin primary cell walls (Fig. 4.3M). At maturity, schizogenous cavities have a layer of epithelial cells surrounding a wide lumen, while schizolysigenous cavities only have a lumen without secretory epithelial cells. Epithelial cells in schizolysigenous cavities have collapsed at some developmental stage and show secretions around the lumen. Secretory cavities are schizogenous in most of the species (e.g., *Amomyrtus* spp., *Myrcianthes coquimbensis*, *Myrceugenia* spp., *M. stipularis*, *Ugni* spp.) (Fig. 4.3N) and schizolysigenous in others (e.g., *B. cruckshanksii*, *L. chequen*, *Myrteola nummularia*,) (Figs 4.3O, 4.3P). A number of species (e.g., *L. chequen*, *Myrcianthes coquimbensis*) have additional secretory cavities throughout palisade and spongy parenchyma (Fig. 4.3P). In surface view, two overlying cells (epidermal cells above secretory cavities) can be observed. These cells vary in shape and are surrounded by a variable number of epidermal cells (Figs 4.2I, 4.2J, 4.2K, 4.2L). The cavities and overlying cells can be clearly differentiated as polyhedral in shape in *Myrceugenia exsucca* (Fig. 4.4B) and *Myrceugenia leptospermoides* (Fig. 4.4A). The overlying cells are barely visible in *Ugni* spp. and *Myrteola nummularia* (Fig. 4.4C). Histochemical reaction with Sudan IV suggests the presence of lipophilic substances in the epithelial cells lining the cavity. Extrafloral nectaries are observed on the adaxial surface of *Myrceugenia planipes* (Fig. 4.4E) and *M. stipularis* (Fig. 4.4F).

Vascular system

Most taxa have a flattened or slightly grooved adaxial leaf surface above the vascular region, but some species possess a noticeable depression (e.g., *L. concinna*, *Myrceugenia exsucca*, *Myrceugenia planipes*, *Ugni molinae*). A prominent swelling on the adaxial side of the leaf

over the main vascular system is observed in *B. cruckshanksii* and *N. fernandeziana*, species that morphologically do not have impressed midribs as the remaining Chilean Myrtaceae.

The vascular system occupies the half of the lamina in cross section in almost all the species, but it is particularly small in *Myrceugenia rufa* (Fig. 4.8H), *Myrteola nummularia* (Fig. 4.8J), *M. stipularis* (Fig. 4.8K) and *U. candollei*. The shape of vascular systems in transverse section varies from circular (*Myrceugenia chrysocarpa, Myrcianthes coquimbensis, Myrteola nummularia, M. stipularis* Figs 4.8E, 4.8I, 4.8J, 4.8K) to arc-shaped vascular systems (e.g., *Myrceugenia correifolia*, *Myrceugenia planipes*, *U. molinae* and *U. selkirkii*- Figs 4.8G, 4.8L). The vascular system is composed of a central region of xylem with bicollateral phloem (adaxial and abaxial) in all the species. The adaxial phloem may be confluent with the abaxial phloem, i.e., merged together to form an arc of continuous phloem, or could be discontinuous and not connected to the abaxial phloem. The adaxial phloem itself could be a single patch (continuous) or it could form two islands of phloem due to the presence of a partition, composed of fibres, vessels or parenchymatous cells. The adaxial phloem partition can be considered either weak or strong depending on the degree of separation between the two patches of adaxial phloem. The amount of adaxial phloem can vary from scarce to abundant, which can be interpreted as poorly and well developed respectively. In the vascular system of *A. luma* and *L. concinna* the adaxial and abaxial phloem is confluent and the adaxial phloem does not have partition, forming a continuous ring that surrounds the xylem (Figs 4.8A, 4.8C). In some species, the adaxial phloem has a weak partition and there is confluence between the adaxial and abaxial phloem (e.g., *Myrceugenia correifolia*, *Myrceugenia exsucca*, *Myrceugenia leptospermoides*). In the remaining species, the adaxial phloem has a strong partition. Some of these have a confluent adaxial and abaxial phloem, such as *A. meli*, *Luma* spp., *Myrceugenia chrysocarpa*, *Myrceugenia obtusa* (Figs 4.8D, 4.8E, 4.8F). Species with a strong adaxial partition and without adaxial-abaxial confluence (vascular system with open extremities) include *Myrceugenia rufa, Myrcianthes coquimbensis, N. fernandeziana, U. candollei* and *U. molinae* (Figs 4.8H, 4.8I, 4.8L). In these species, the adaxial phloem usually forms two islands of phloem that are disconnected from the abaxial phloem. In the vascular system of *L. chequen*, *Myrceugenia chrysocarpa*, *Myrceugenia rufa*, *Myrteola nummularia* and *M. stipularis* the adaxial phloem is scarce, unlike the majority of taxa, which have abundant and well developed adaxial phloem (Fig. 4.8). The vascular system of *B. cruckshanksii* has the adaxial phloem curved inward forming two isolated groups of xylem surrounded by adaxial phloem (Fig. 4.8B), while in the case of *N. fernandeziana* the xylem

surrounds two islands of adaxial phloem. The latter can be classified as a vascular system with adaxial phloem with weak partition and without adaxial and abaxial phloem confluence. Sclerenchyma fibres form a continuous ring around the vascular system in the majority of species (Fig. 4.8), but they are discontinuous and form an abaxial arc in *A. meli, Myrceugenia colchaguensis* and *Myrceugenia schulzei.* There are no fibres around the vascular system of *Myrteola nummularia* (Fig. 4.8J) and they are very abundant around the midrib of *Myrcianthes coquimbensis* (Fig. 4.8I). Histochemical reaction to Phloroglucinol+ 20% HCl was observed in all sclerenchymatous tissues, especially fibres in the vascular system.

4.3.2 Synopsis of leaf anatomical characters in genera of Chilean Myrtaceae

The following section is a synopsis of the salient anatomical and micromorphological characters of each genus of Chilean Myrtaceae. For each genus, the species studied are indicated, as well as the total number of accepted species for that genus. Vouchers and herbarium are indicated in parentheses. In this summary, only those characters that were present have been included and the absence of characters is only reported in cases where our observation contradict those already published.

1. *Amomyrtus* (Burret) D.Legrand and Kausel (Figs 4.1A, 4.2A, 4.3D,J, 4.5G, 4.6A, 4.7A).

Number of species in genus: Two

Species studied: *Amomyrtus luma* (H.Retamales 11819-11820 (EIF)), *A. meli* (H.Retamales 11789-11799 (EIF)).

The leaves are hypostomatic with anomocytic stomatal complexes. The epidermis is single-layered, mucilaginous and has a thin cuticle layer (3µm thick or less). The adaxial epidermis is slightly thicker than the abaxial epidermis. Epidermal anticlinal walls are highly sinuous and thin. The leaves are glabrous to sparsely pubescent on midrib and margins, but more pubescent in *A. luma* than *A. meli*. The hairs are simple and straight-curved. The mesophyll is dorsiventral with two-three layers of palisade parenchyma. The spongy parenchyma is composed of large, isodiametric cells. Idioblasts containing druses are distributed only below the adaxial epidermis. The secretory cavities are schizogenous and are mainly located below the adaxial epidermis. The shape of the vascular system is circular. The adaxial phloem is abundant and continuous (without partition) in *A. luma*, while it is partitioned into two clear clusters in *A. meli*. The adaxial and abaxial phloem is confluent in *A. luma* but not so in *A. meli*. Fibres form a continuous ring around the vascular system.

2. *Blepharocalyx* O.Berg (Figs 4.1B, 4.2E,I, 4.7B).

Number of species in genus: Three

Species studied: *B. cruckshanksii* (H.Retamales 11803 (EIF))

The leaves are hypostomatic with anomocytic stomatal complexes. The epidermis is single-layered, mucilaginous and has a regular cuticle layer (3-5µm thick). The adaxial epidermis is slightly thicker than the abaxial epidermis. The epidermal cells are isodiametric with highly sinuous and thin anticlinal walls on the abaxial surface. The leaves are glabrous. The mesophyll is dorsiventral with two-three layers of palisade parenchyma. The spongy parenchyma is composed of small, irregularly shaped cells. Idioblasts containing druses are distributed below the adaxial epidermis. The secretory cavities are schizolysigenous and are mainly located below the adaxial epidermis. The shape of the vascular system is ellipsoidal. The adaxial phloem is abundant with a weak partition and surrounds two islands of xylem. The adaxial and abaxial phloem is not confluent. Fibres are discontinuous around the vascular system.

3. *Legrandia* Kausel (Figs 4.1C, 4.3L, 4.4D, 4.7B, 4.8C).

Number of species in genus: 1

Species studied: *L. concinna* (H.Retamales 11821 (EIF))

The leaves are hypostomatic with anomocytic stomatal complexes. The epidermis is single-layered, mucilaginous and has a regular cuticle layer (3-5µm thick). The adaxial epidermis is slightly thicker than the abaxial epidermis. The epidermal cells are isodiametric with highly sinuous and thin anticlinal walls on the abaxial surface. Domatia are observed in the axils of veins on the abaxial surface. Conical papillae are present on the abaxial surface. The leaves are glabrous to sparsely pubescent on midrib and margins. The hairs are simple and straight-curved. The mesophyll is dorsiventral with three layers of palisade parenchyma. The spongy parenchyma is composed of large, isodiametric cells. Idioblasts containing druses are distributed below the adaxial epidermis, sometimes forming clusters of six-seven. The secretory cavities are schizogenous and are mainly located below the adaxial epidermis. The shape of the vascular system is arc-shaped. The adaxial phloem is abundant and continuous (without partition). The adaxial and abaxial phloem is confluent. Fibres are discontinuous around the vascular system.

4. *Luma* Gray (Figs 4.1D, 4.2F, 4.3A,F,G,P, 4.5I, 4.8D).

Number of species in genus: 2

Species studied: *L. apiculata* (H.Retamales 11811 (EIF)), *L. chequen* (H.Retamales 11812 (EIF)).

The leaves are hypostomatic with paracytic stomatal complexes. The epidermis is single-layered, mucilaginous and has a regular cuticle layer (3-5µm thick). The adaxial epidermis is slightly thicker than the abaxial epidermis. The epidermal cells are isodiametric with slightly sinuous and thin anticlinal walls. The leaves are glabrous to sparsely pubescent on midrib and margins. The hairs are simple and straight-curved in *L. apiculata*, while *L. chequen* also has glandular hairs on both surfaces. The mesophyll is dorsiventral with two-three layers of palisade parenchyma in *L. apiculata* and two layers in *L. chequen*. The spongy parenchyma is composed of small, isodiametric cells. Idioblasts containing druses are distributed only below the adaxial epidermis. The secretory cavities are schizogenous and mainly located below the adaxial epidermis in *L. apiculata* and schizolysigenous and located throughout the mesophyll in *L. chequen*. The shape of the vascular system is ellipsoidal. The adaxial phloem is abundant with a strong partition in *L. apiculata* and scarce with a weak partition in *L. chequen*. The adaxial and abaxial phloem is confluent in *L. apiculata* and not confluent in *L. chequen*. Fibres are discontinuous around the vascular system.

5. *Myrceugenia* O.Berg (Figs 4.1E-H, 4.2D,K, 4.3C,E,I,N, 4.4A,B,E,H,I, 4.5A-C,E,H, 4.6B,D,F, 4.7A,C,D, 4.8E-H).

Number of species in genus: ca. 40

Species studied: *M. chrysocarpa* (H.Retamales 11796 (EIF)), *M. colchaguensis* (Crawford and Baeza 116898 (CONC)), *M. correifolia* (H.Retamales 11807 (EIF)), *M. exsucca* (H.Retamales 11815 (EIF)), *M. lanceolata* (H.Retamales 11818 (EIF)), *M. leptospermoides* (H.Retamales 11821 (EIF)), *M. obtusa* (H.Retamales 11800 (EIF)), *M. ovata var. ovata* (H.Retamales 11801 (EIF)), *M. ovata* var. *nanophylla* (Crawford and Baeza 157851 (CONC)), *M. parvifolia* (H.Retamales 11810 (EIF)), *M. pinifolia* (Crawford and Baeza 157850 (CONC)), *M. planipes* (H.Retamales 11802 (EIF)), *M. rufa* (H.Retamales 11813 (EIF)), *M. schulzei* (H.Retamales 11814 (EIF)).

The leaves are hypostomatic in all species. The stomatal complexes are anomocytic in most of the species, but actinocytic (and anomocytic) in *M. colchaguensis*. The epidermis is single-layered in all the species except for the adaxial epidermis of *M. rufa*, where the epidermis is double-layered. The epidermis is mucilaginous and has a regular cuticle layer (3-5µm thick) in most of the species, but thick (>5µm, up to 8µm) in *M. correifolia* and *M. rufa*. The adaxial epidermis is slightly thicker than the abaxial in most of the cases but thicker and equally thick in *M. obtusa*. The epidermal cells are isodiametric with highly sinuous and thin anticlinal walls on the abaxial surface in most of the species but highly sinuous in *M. pinifolia* and straight and thick in *M. colchaguensis, M. correifolia* and *M. rufa.* Conical papillae present on the abaxial surface of *M. correifolia* and *M. schulzei*. All the species possess dibrachiated hairs. Simple hairs were observed in *M. lanceolata, M. leptospermoides, M. obtusa, M. ovata, M. parvifolia* and *M. pinifolia*. Glandular hairs were only seen in *M. colchaguensis* and *M. obtusa*. The mesophyll is dorsiventral with two-three layers of palisade parenchyma, except for *M. parvifolia* that possess only one layer. The spongy parenchyma has isodiametric or irregularly shaped cells, with abundant intercellular spaces in most species and scarce in *M. correifolia* and *M. rufa*. Idioblasts containing druses are observable below the adaxial epidermis in most species, but also occur around the vascular system in *M. colchaguensis* and *M. schulzei.* Most of the species have druses, but spherical crystals are observed in *M. chrysocarpa* and rhombohedral in *M. colchaguensis* and *M. schulzei*. The secretory cavities are schizogenous in all the species, except for *M. correifolia, M. obtusa* and *M. ovata*, which have schizolysigenous cavities. The vascular system is arc-shaped in all the species other than *M. chrysocarpa* in which it is circular. The adaxial phloem is either scarce or abundant and the partition weak or strong depending upon species (Table 4.2). The adaxial and abaxial phloem is confluent in most of taxa, but not confluent in *M. lanceolata*, *M. ovata, M. rufa* and *M. schulzei*. Fibres are discontinuous around the vascular system.

6. *Myrcianthes* Berg (Figs 4.1I, 4.2H, 4.5D, 4.6E, 4.7F, 4.8I).

Number of species in genus: 30

Species studied: *M. coquimbensis* (H.Retamales 11822 (EIF)).

The leaves are hypostomatic with laterocytic (and paracytic) stomatal complexes. The epidermis is single-layered, mucilaginous and has a tick cuticle layer (>5µm, up to 8µm thick). The adaxial and abaxial epidermises are tick and have the same thickness. The epidermal cells are isodiametric with slightly sinuous anticlinal walls on the abaxial surface.

The leaves are densely covered by hairs in both abaxial and adaxial surfaces. The hairs are simple and straight-curved. Glandular hairs are also observed. The mesophyll is dorsiventral with two layers of palisade parenchyma. The spongy parenchyma is composed of large, irregularly shaped cells. Idioblasts containing druses are distributed below the adaxial epidermis. The secretory cavities are schizogenous and are located below the adaxial epidermis and throughout the mesophyll. The shape of the vascular system is circular. The adaxial phloem has a medium development (abundance) and a strong partition. The adaxial and abaxial phloem is confluent. Fibres form a continuous ring around the vascular system.

7. *Myrteola* Berg (Figs 4.1J, 4.2C, 4.3O, 4.4C, 4.8J).

Number of species in genus: 3

Species studied: *M. nummularia* (H.Retamales 11804 (EIF)).

The leaves are amphistomatic with anomocytic stomatal complexes. The epidermis is single-layered, mucilaginous and has a thin cuticle layer (3µm thick or less). The adaxial epidermis is thicker than the abaxial epidermis. The epidermal cells are isodiametric with highly sinuous and thin anticlinal walls on the abaxial surface. The leaves are glabrous to sparsely pubescent on midrib and margins. The hairs are simple and straight-curved. The mesophyll is dorsiventral with two-three layers of palisade parenchyma. The spongy parenchyma is composed of large, isodiametric cells. Idioblasts containing druses are distributed below the adaxial epidermis. The secretory cavities are schizolysigenous and are mainly located below the adaxial epidermis. The shape for the vascular system is circular. The adaxial phloem is scarce and continuous (without partitions). The adaxial and abaxial phloem is confluent. There are no fibres around the vascular system.

8. *Nothomyrcia* Kausel

Number of species in genus: 1

Species studied: N. fernandeziana (H.Retamales 11816 (EIF)).

The leaves are hypostomatic with anomocytic stomatal complexes. The epidermis is single-layered, mucilaginous and has a regular cuticle layer (3-5µm thick). The adaxial epidermis is slightly thicker than the abaxial epidermis. The epidermal cells are isodiametric with slightly sinuous and thin anticlinal walls on the abaxial surface. A hypodermis is observed under the adaxial epidermis. The leaves are glabrous to sparsely pubescent on midrib and margins. The

hairs are simple and straight-curved. The mesophyll is dorsiventral with two-three layers of palisade parenchyma. The spongy parenchyma is composed of large, irregularly shaped cells. Idioblasts containing druses are distributed below the adaxial epidermis. The secretory cavities are schizogenous and are mainly located below the adaxial epidermis. The shape of the vascular system is ellipsoidal. The adaxial phloem is abundant with a weak partition. Xylem and fibres surrounds two islands of adaxial phloem. The adaxial and abaxial phloem is not confluent. Fibres form a continuous ring around the vascular system.

9. *Ugni* Turcz. (Figs 4.1L, 4.2G, 4.3K,M, 4.4G, 4.5F, 4.6C, 4.8L)

Number of species in genus: 4

Species studied: *U. candollei* (H.Retamales 11806 (EIF)), *U. molinae* (H.Retamales 11808 (EIF)), *U. selkirkii* (T.Stuessy and Crawford 121491 (CONC)).

The leaves are hypostomatic with anomocytic stomatal complexes in *U. molinae* and *U. selkirkii*, but anisocytic (and anomocytic) in *U. candollei*. The epidermis is single-layered, mucilaginous and has a regular cuticle layer (3-5µm thick). The adaxial epidermis is slightly thicker than the abaxial epidermis. The epidermal cells are isodiametric with slightly sinuous and thin anticlinal walls. A hypodermis is observed under the adaxial epidermis in *U. candollei*. The leaves are glabrous to sparsely pubescent on midrib and margins in *U. molinae* and *U. selkirkii*. *Ugni candollei* have sparse to moderately pubescent leaves particularly on midribs. The hairs are simple and straight-curved in *U. molinae* and *U. selkirkii*, while *U. candollei* also has dibrachiate hairs. The mesophyll is dorsiventral with two-three layers of palisade parenchyma in *U. candollei and U. selkirkii*, while in *U. molinae* three layers are observed. The spongy parenchyma is composed of small, irregularly shaped cells. Idioblasts containing druses are observable below the adaxial epidermis in *U. candollei* and *U. molinae*, but also occur around the vascular system in *U. selkirkii.* Rhombohedral crystals are observed in *U. selkirkii*. The secretory cavities are schizogenous and mainly located below the adaxial epidermis. The vascular system is arc-shaped with strong curvature in *U. molinae* and *U. selkirkii*, while it is circular in *U. candollei*. The adaxial phloem has a medium development and has a strong partition in *U. candollei* and *U. molinae*, while there is a weak partition in *U. selkirkii*. The adaxial and abaxial phloem is not confluent in *U. candollei* and *U. molinae*, but it is confluent in *U. selkirkii*. Fibres are discontinuous around the vascular system.

10. *Metrosideros* Banks ex Gaertn. (Figs. 4.1K, 4.2L, 4.4F, 4.8K).

Number of species in genus: 1

Species studied: *M. stipularis* (H.Retamales 11805 (EIF)).

The leaves are hypostomatic with anomocytic stomatal complexes. The transverse section of the leaf is ellipsoid-shaped. The epidermis is single-layered, mucilaginous and has a thin cuticle layer (3µm thick or less). The adaxial epidermis is thicker than the abaxial epidermis. The epidermal cells are isodiametric with slightly sinuous and thin anticlinal walls on the abaxial surface. The leaves are glabrous to sparsely pubescent on midrib and margins. The hairs are simple and straight-curved. The mesophyll is dorsiventral with two-three layers of palisade parenchyma. The spongy parenchyma is composed of large, irregularly shaped cells. Crystals were not found in the species. The secretory cavities are schizolysigenous and are mainly located below the adaxial epidermis. The shape of the vascular system is circular. The adaxial phloem is scarce, with a weak partition. The adaxial and abaxial phloem is not confluent. Fibres are discontinuous around the vascular system, forming a prominent plate under the abaxial phloem.

4.3.3 Identification key

The following identification key is based on leaf morpho-anatomical characters for genera and species of Chilean Myrtaceae (*Myrceugenia* species not included).

1. Amphistomatic leaves…….…….……***Myrteola nummularia***

1. Hypostomatic leaves……..2

2. Presence of domatia on abaxial surface……..***Legrandia concinna***

2. Absence of domatia on abaxial surface…….…….3

3. Transverse section of leaf ellipsoid-shaped, no crystals in leaves……....***Metrosideros stipularis***

3. Transverse section of leaf other than above, crystals in leaves.…….……4

4. Leaves with a pronounced parenchymatous swelling over midrib……..……..5

4. Leaves with depression above midrib……..……...……...6

5. Hypodermis present, adaxial xylem surrounding two islands of phloem…….....…....***Nothomyrcia fernandeziana***

5. Hypodermis absent, adaxial phloem surrounding two islands of xylem……..…….….***Blepharocalyx cruckshanksii***

6. Leaves with paracytic stomata (Fig. 4.2F)……7 (***Luma***)

6. Leaves with stomata other than paracytic…….……8

7. Glandular hairs present, schizolysigenous cavities throughout mesophyll.....***Luma chequen***

7. Glandular hairs absent, schizogenous cavities under adaxial epidermis…….***Luma apiculata***

8. Arc-shaped vascular system (Fig. 4.8G)…..……..……...……9

8. Shape of vascular system other than arc…….……..……..….10

9. Dibrachiate hairs present (Fig. 4.6B)……..…… ***Myrceugenia***

9. Dibrachiate hairs absent…….……..13

10. Laterocytic stomata, glandular hairs present, epidermis thick on both surfaces, epidermal cells with 1:1 size ratio……..……..… ***Myrcianthes coquimbensis***

10. Stomata other than laterocytic, glandular hairs absent, epidermis thin, usually thicker on the adaxial surface…….……...11

11 Hypodermis present, conical papillae present, anisocytic stomata….……***Ugni candollei***

11. Hypodermis absent, papillae absent, anomocytic stomata ……...….12 (***Amomyrtus***)

12. Continuous adaxial phloem in vascular systems (Fig. 4.8A)……..…....***A. luma***

12. Partitioned adaxial phloem in vascular systems……..……..…....***A. meli***

13. Druses under adaxial epidermis, strong adaxial phloem partition……..…….***U. molinae***

13. Prismatic rhombohedral crystals around vascular system, weak adaxial phloem partition.....***U. selkirkii***

4.4 Discussion

A number of the leaf anatomical and micromorphological characters observed here can be used to identify genera or species. The anatomical results of this investigation largely agree with those for South American Myrtaceae in Fontenelle et al. (1994), Donato and Morretes (2009, 2011), Cardoso et al. (2009), Gomes et al. (2009) and Soh and Parnell (2011). Differences in some characters were observed and will be pointed out in this discussion. Potential links between anatomical characters and environmental conditions are also discussed.

4.4.1 Epidermis and indumentum

Here we have interpreted the hypodermis as a layer of large cells located below a single layer of smaller epidermal cells and mainly originated from the ground meristem (Martins et al., 2012). On the other hand, two or more layers of aligned cells and originated from the protodermis were considered a multiple epidermis (Dickison, 2000; Sharma and Mehra, 1972; Martins et al., 2012). The hypodermis and multiple epidermis are regarded as two non-homologous anatomical features; therefore, ontogenetic observations are always recommended to avoid misinterpretations (Martins et al., 2012). The presence of a multiple epidermis or hypodermis has been considered an ecological adaptation of xerophytic plants to arid environments, which prevents water loss due to excessive evapotranspiration and protects the lamina from high solar radiation (Dickison, 2000; Metcalfe and Chalk, 1979; Evert, 2006). A single epidermis is commonly associated with mesophytic and hydrophytic species and is considered the normal type of epidermis in vascular plants (Dickison, 2000). The presence of a single epidermis has been reported for most species of the family Myrtaceae (Metcalfe and Chalk, 1979). Genera with single-layered epidermis include *Eugenia* (Armstrong et al., 2012; Donato and Morretes, 2009; Fontenelle et al., 1994), *Myrcia, Campomanesia* (Gomes et al., 2009), *Callistemon, Eucalyptus, Melaleuca* (Tantawy, 2004), *Acmena*, *Syzygium*, *Heteropyxis,* and *Tristania* (Keating, 1984; Soh and Parnell, 2011). The presence of a hypodermis has been identified in *Campomanesia*, *Myrcianthes*, *Psidium* and *Pimenta* (Cardoso et al., 2009; Gomes et al., 2009). Cardoso et al. (2009) reported the presence of hypodermis in the Brazilian species *Myrceugenia euosma*. *Myrceugenia rufa* is the only species of Chilean Myrtaceae with adaxial double epidermis and can be reliably identified using this anatomical character. The main habitat of *Myrceugenia rufa* is the xeromorphic shrublands of north-central Chile, where rainfall is restricted to few days of the year (Serra et al., 1986). The presence of double epidermis in this species supports this ecological association. The occurrence of an adaxial hypodermis was observed only in *N. fernandeziana* and *U. candollei*, species that mainly occur in wet forests and open vegetation in humid regions of Chile. In this case, the presence of hypodermis might not be associated with a xerophytic habitat. *Nothomyrcia fernandeziana* is phylogenetically positioned within a clade that is closely related to the "*Pimenta* group" (Murillo et al., 2013), which includes genera known to have hypodermis, such as *Pimenta* and *Psidium* (Cardoso et al., 2009). Consequently, the presence of hypodermis in *Nothomyrcia*, *Pimenta* and *Psidium* could be due to phylogenetic history and not

environment. As the systematic position of *U. candollei* is unknown, the presence of hypodermis cannot yet be linked to phylogenetic constraints.

Papillae have been reported as projections of the epidermal cells in some Myrtaceae, including South American species such as *Gomidesia nitida* and *Myrceugenia euosma* (Cardoso et al., 2009; Metcalfe and Chalk, 1979). Here we observed papillae on the leaf surface of *L. concinna*, *Myrceugenia correifolia*, *Myrceugenia schulzei* and *U. candollei*, species that occur in distinct environments (mesophytic and xerophytic). The role of papillae needs more investigation, but might be related to plant defence against pathogens and herbivory (Voigt, 2014).

The anticlinal epidermal walls correspond to the outline of the primary walls between adjacent cells and depend on the cellulose microfibril organization and deposition (Panteris et al., 1993). Epidermal anticlinal walls have low intraspecific variation in Myrtaceae (Carr et al., 1971) and can be regarded as a taxonomically stable character (Pole, 2010). The shape of anticlinal epidermal walls is considered an environmental adaptation, as mesophytic species usually have sinuous walls while xerophytic have straight walls (Gifford and Foster, 1989). Fontenelle et al. (1994) have reported straight and thick epidermal anticlinal walls in xerophytic species of *Eugenia*. Our observations of the Chilean Myrtaceae support these environmental associations as those species occurring in xerophytic habitats (*Myrceugenia correifolia*, *Myrceugenia rufa*, *Myrcianthes coquimbensis*) have straight anticlinal walls, while mesophytic species possess slightly sinuous or highly sinuous walls. Epidermal anticlinal walls (mainly abaxial) are a suitable character for delimiting a number of species of Chilean Myrtaceae.

The occurrence of hairs in plants is regarded as a xerophytic adaptation, especially when the hair covering is dense (Evert, 2006). Hairs extend the boundary layer in a leaf, which creates a stable microclimate on the surface and reduces water losses due to excessive solar radiation (Ehleringer, 1985). Fontenelle et al. (1994) suggest that some xerophytic characters in Myrtaceae (straight anticlinal walls, hairs, waxes) are not strictly associated with environmental conditions, as species from different geographic zones and habitats, encompassing xerophytic and mesophytic habitats, possess these features. Leaves of Myrtaceae are often glabrous or possess scattered hairs on midribs and leaf blades (Wilson, 2011). Unicellular hairs are the main type of trichome present in Myrtaceae (Briggs and Johnson, 1979; Metcalfe and Chalk, 1979) and the only type found in Chilean species.

Trichomes observed in Chilean Myrtaceae largely agree with the results reported by Landrum (1981b, 1986, 1988a). Simple hairs are widely present in South American Myrtaceae (Cardoso et al., 2009; Gomes et al., 2009) and were observed here in *Amomyrtus*, *Legrandia*, *Luma*, *Myrcianthes*, *Myrteola*, *Nothomyrcia* and two species of *Ugni*. Dibrachiate hairs (armed biramous hairs) were observed in all the species of Chilean *Myrceugenia* and also in *U. candollei*. Most of the species of *Myrceugenia* are reported to possess dibrachiate hairs, as well as *Calyptranthes*, *Eugenia*, *Marlierea* and some species of *Myrcia* (Landrum and Kawasaki, 1997). The presence of glandular or secretory hairs is not widely reported in South American Myrtaceae. Secretory hairs have been reported on the abaxial leaf surface of the Brazilian species *Myrceugenia euosma*, formed by papillose cells with thick cell walls (Cardoso et al., 2009). Wilson (2011) refers to infundibular hairs (funnel-shaped) in a group of South American *Eugenia*, but such hairs were not observed in any Chilean species. The dense layer of hairs on the abaxial leaf surface was observed in a number of Chilean species from arid environments (*Myrcianthes coquimbensis*, *Myrceugenia colchaguensis*, *Myrceugenia correifolia* and *Myrceugenia rufa*). Most of these species occur in coastal shrublands in the north-centre of Chile (Landrum, 1988a), where rainfall and humidity are much lower compared to the typical mesophytic habitat of Chilean Myrtaceae. *Myrceugenia euosma* is a South American species that occurs in Mata Atlântica and the states of São Paulo, Paraná, Santa Catarina and Rio Grande do Sul (Sobral et al., 2015) and that has been considered one of the most xerophytic species of the genus (Landrum, 1981b). Although *Myrceugenia euosma* resembles the xerophytic *Myrceugenia rufa*, the first species has been reported to occur also in flooded environments (Cardoso et al., 2009). In order to confirm the consistency of some anatomical characters/character states related to ecological and environmental associations (e.g., hypodermis, epidermal anticlinal walls, hairs), comprehensive sampling of more populations is recommended. The phylogenetic position of the most pubescent species of Chilean *Myrceugenia* is either sister to the rest of the genus (*Myrceugenia rufa*) or part of a monophyletic group near the base (*Myrceugenia colchaguensis* + *Myrceugenia schulzei*) (Murillo et al., 2013). In order to infer whether trichome characters have a common phylogenetic origin or are product of convergent evolution, further investigation is required.

4.4.2 Stomata

Although distribution of stomata and types of stomatal complexes are considered important for taxonomic delimitation, there are a number of different classifications, each with a

particular terminology (Dressler, 1993). For a better understanding of stomatal complexes, ontogenetic studies are critically important (Carpenter, 2005; Pole, 2010). Developmental or ontogenetic studies are also necessary to find out if different types of mature stomata are homologous (Pole, 2010).

Amphistomatic leaves (stomata distributed on both abaxial and adaxial leaf surfaces) are commonly observed in hydrophytes and creeping species from wet habitats (Evert, 2006; Gifford and Foster, 1989). It has been reported that some exceptions are sclerophyll species with isobilateral leaf anatomy that might have amphistomatic leaves, including some *Eucalpytus* species (Tantawy, 2004). The presence of amphistomatic leaves in *Myrteola nummularia* suggests an environmental correlation with the habitat of the species. In Chile, *Myrteola nummularia* is mainly a creeping shrub or sub-shrub that occurs in wet habitats such as swamps, peatlands and the lower strata of humid forests (Landrum, 1988b).

Eugenia is one of the most widely studied genera of Myrtaceae and paracytic stomatal complexes the most common type in the genus (Fontenelle et al., 1994; Haron and Moore, 1996; Hussin et al., 1992). Anomocytic stomata have also been reported as a common type at family level (Gomes et al., 2009; Metcalfe and Chalk, 1979). Paracytic stomata were observed only in the two species of the genus *Luma*, while the anomocytic type was observed in a number of genera (*Amomyrtus*, *Legrandia*, *Myrceugenia*, *Ugni*). The different types of stomatal complexes observed in Chilean Myrtaceae can be used to some extent to delimit genera.

4.4.3 Mesophyll, crystals and secretory cavities

Dorsiventral (bifacial) mesophyll is the most common type of mesophyll in Myrtales and Myrtaceae (Keating, 1984; Wilson, 2011). Few genera, such as the Australasian *Corymbia*, *Eucalyptus*, *Leptospermum* and *Melaleuca*, species with vertically oriented leaves, have isobilateral mesophyll (Gomes et al., 2009; Wilson, 2011). All the Chilean Myrtaceae have dorsiventral mesophyll and the leaves are generally horizontally positioned. Mucilage and pectins were stained by ruthenium red as granules or red content in the mesophyll of all species, as indicated by Jensen (1962).

Crystals composed of calcium oxalate are the most common biomineral occurring in plants (Arnott, 1982). These structures have been related to the regulation of calcium activity in tissues (Volk et al., 2002), as well as protection against herbivores and pathogens (Franceschi

and Nakata, 2005). Calcium oxalate crystals are widely present in Myrtaceae and have different shapes and structure (Metcalfe and Chalk, 1979). Druses are the most common type of crystal in Chilean Myrtaceae and have been also reported in *Eugenia*, *Gomidesia*, *Psidium* and *Myrcia*, among other South American genera (Cardoso et al., 2009; Gomes et al., 2009). Rhombohedral crystals observed in *Myrceugenia colchaguensis*, *Myrceugenia schulzei* and *Ugni selkirkii* are similar to those reported for the Australasian genus *Syzygium* (Soh and Parnell, 2011) and other South American genera, such as *Calyptranthes*, *Campomanesia*, *Gomidesia* and *Mosiera* (Cardoso et al., 2009).

Schizogenous secretory cavities are originated by separation of cells and are composed of a layer of epithelial cells surrounding a wide lumen space at maturity (Ciccarelli et al., 2008). Lysigenous secretory cavities arise by dissolution of cells and do not possess epithelial cells at maturity (Evert, 2006). Schizolysigenous cavities occur when cavities arise due to the separation of cells (schizogenous origin), but epithelial cells are dissolved at maturity by autolysis (Evert, 2006). Secretory cavities are mainly located adjacent to the adaxial and/or abaxial epidermis and are primarily protodermal in origin, with participation of the ground meristem (Arruda and Fontenelle, 1994; Fahn, 1979). The role of compounds produced by secretory cavities (mainly sesquiterpenes and flavonoids in Myrtaceae) has been associated to a number of plant functions. These roles are related to direct defence responses, metabolism of diverse chemicals (Banthorpe et al., 1972) and plant architecture, through inhibition of shoot branching (Akiyama et al., 2008). Secretory cavities are one of the most distinctive features of Myrtaceae (Wilson et al., 2011), and are often referred as oil dots in field guides and keys. Schizogenous secretory cavities are the most common type observed in Myrtaceae (Alves et al., 2008; Donato and Morretes, 2011; Gomes et al., 2009) and also in Chilean species. Schizolysigenous cavities were observed in a few Chilean species.

4.4.4 Vascular system

All species of Chilean Myrtaceae, other than *Nothomyrcia fernandeziana*, have been described as possessing leaves with impressed midribs (Landrum, 1988a). Anatomically, the pronounced swelling above the midrib of *N. fernandeziana* is composed of large and isodiametric parenchymatous cells. *Blepharocalyx cruckshanksii* possess a slight swelling above the midrib, which is not usually reported in morphological descriptions of the species. Adaxial phloem in vascular system is regarded as a typical character in the order Myrtales (Cronquist, 1981) and is widely present in Myrtaceae (Cardoso et al., 2009; Schmid, 1980).

Vascular system characters observed here, such as adaxial phloem partition, confluence of adaxial and abaxial phloem and sclerenchyma (fibres) around the vascular system, largely agree with what has been observed in other South American genera (Cardoso et al., 2009; Donato and Morretes, 2009; Gomes et al., 2009). These features are considered suitable characters to identify species in Myrtaceae (Cardoso et al., 2009; Soh and Parnell, 2011). *Blepharocalyx cruckshanksii* and *N. fernandeziana* are the only species of Chilean Myrtaceae with inwardly curved vascular tissues: phloem surrounding two islands of xylem in *B. cruckshanksii* and xylem surrounding phloem in *N. fernandeziana*. This anatomical character supports the close phylogenetic relationship suggested for these two species and the recognition of *Nothomyrcia*, as a separate genus distinct from *Myrceugenia* (Murillo and Ruiz, 2011; Murillo et al., 2013).

4.4 Conclusion

This is the first investigation that describes the leaf anatomy of the 26 species of Chilean Myrtaceae, including all the accepted species of a number of genera (*Amomyrtus*, *Legrandia*, *Luma*). Anatomical features described here largely agree with previous characters found in other Myrtaceae. Most of the species possess a typical mesophytic leaf anatomy, while others show a combination of xerophytic characters such as hairy leaves, hypodermis, thick adaxial epidermis and straight epidermal anticlinal walls. Anatomical and micromorphological characters described here have potential taxonomic, ecologic and phylogenetic significance. Yet, anatomical descriptions of other South American and Australasian genera of Myrteae are recommended in order to use these features in a broader taxonomic and evolutionary context. Further anatomical studies from additional populations are recommended in order to confirm the consistency of some characters at species level.

Table 4.1. Leaf anatomical and micromorphological characters in epidermis of Chilean Myrtaceae

	Epidermis and papillae			Stomata	Indumentum
Taxon	Epidermis	Sinuosity of abaxial anticinal walls	Papillae	Stomatal type	Type of hairs
Amomyrtus luma (Molina) D. Legrand and Kausel	Single	High	Absent	Anomocytic	Simple
Amomyrtus meli (Phil.) D. Legrand and Kausel	Single	High	Absent	Anomocytic	Simple
Blepharocalyx cruckshanksii (Hook. and Arn.) Nied.	Single	High	Absent	Anomocytic	Absent
Legrandia concinna (Phil.) Kausel	Single	High	Conical	Anomocytic	Simple
Luma apiculata (DC.) Burret	Single	High	Absent	Paracytic	Simple
Luma chequen (Feuillée ex Molina) Gray	Single	Slight	Absent	Paracytic	Simple and glandular
Myrceugenia chrysocarpa (O.Berg) Kausel	Single	Slight	Absent	Anomocytic	Dibrachiate
Myrceugenia colchaguensis (Phil.) Navas	Single	Straight	Absent	Actinocytic (anomocytic)	Dibrachiate and glandular
Myrceugenia correifolia (Hook. and Arn.) O.Berg	Single	Straight	Conical	Anomocytic	Dibrachiate

	Epidermis and papillae			Stomata	Indumentum
Taxon	Epidermis	Sinuosity of abaxial anticinal walls	Papillae	Stomatal type	Type of hairs
Myrceugenia exsucca (DC.) O.Berg	Single	Slight	Absent	Anomocytic	Dibrachiate
Myrceugenia lanceolata (Juss. ex J. St.-Hil.) Kausel	Single	Slight	Absent	Anomocytic	Simple and dibrachiate
Myrceugenia leptospermoides (DC.) Kausel	Single	Slight	Absent	Anomocytic	Simple and dibrachiate
Myrceugenia obtusa (DC.) O.Berg	Single	Slight	Absent	Anomocytic	Simple, dibrachiate and glandular
Myrceugenia ovata (Hook. and Arn.) O.Berg	Single	Slight	Absent	Anomocytic	Simple and dibrachiate
Myrceugenia ovata var. nanophylla (Burret) L.R. Landrum	Single	Slight	Absent	Anomocytic	Simple and dibrachiate
Myrceugenia parvifolia (DC.) Kausel	Single	Slight	Absent	Anomocytic	Simple and dibrachiate
Myrceugenia pinifolia (F. Phil.) Kausel	Single	High	Absent	Anomocytic	Simple and dibrachiate
Myrceugenia planipes (Hook. and Arn.) O.Berg	Single	Slight	Absent	Anomocytic	Dibrachiate
Myrceugenia rufa (Colla) Skottsb. ex Kausel	Double	Straight	Absent	Anomocytic	Dibrachiate
Myrceugenia schulzei Johow	Single	Slight	Conical	Anomocytic	Dibrachiate
Myrcianthes coquimbensis (Barnéoud) Landrum and Grifo	Single	Slight	Absent	Laterocytic	Simple and glandular

	Epidermis and papillae			Stomata	Indumentum
Taxon	Epidermis	Sinuosity of abaxial anticinal walls	Papillae	Stomatal type	Type of hairs
				(paracytic)	
Myrteola nummularia (Poir.) O.Berg	Single	Slight	Absent	Anomocytic	Simple
Nothomyrcia fernandeziana (Hook. and Arn.) Kausel	Hypodermis	Slight	Absent	Anomocytic	Simple
Ugni candollei (Barnéoud) O.Berg	Hypodermis	Slight	Conical	Anisocytic (anomocytic)	Simple and dibrachiate
Ugni molinae Turcz.	Single	Slight	Absent	Anomocytic	Simple
Ugni selkirkii (Hook. and Arn.) O.Berg	Single	Slight	Absent	Anomocytic	Simple
Metrosideros stipularis (Hook. and Arn.) Griseb.	Single	Slight	Absent	Anomocytic	Absent

Table 4.2. Leaf anatomical characters in the mesophyll and vascular system of Chilean Myrtaceae

	Mesophyll				Vascular system		
Taxon	P.p. layers	Type of crystals	Type of cavities	Shape	Ad. Phloem partition	Phoem confluence	Amount of ad. phloem
Amomyrtus luma (Molina) D. Legrand and Kausel	2-3	Druses	Schizogenous	Ellipsoid	Absent	Confluent	Abundant
Amomyrtus meli (Phil.) D. Legrand and Kausel	2-3	Druses	Schizogenous	Ellipsoid	Strong	Confluent	Medium
Blepharocalyx cruckshanksii (Hook. and Arn.) Nied.	2-3	Druses	Schizolysigenous	Ellipsoid	Weak	Not confluent	Abundant
Legrandia concinna (Phil.) Kausel	3	Druses	Schizogenous	Slight arc	Absent	Confluent	Medium
Luma apiculata (DC.) Burret	2-3	Druses	Schizogenous	Ellipsoid	Strong	Confluent	Medium
Luma chequen (Feuillée ex Molina) Gray	2	Druses	Schizolysigenous	Ellipsoid	Weak	Not confluent	Scarce
Myrceugenia chrysocarpa (O.Berg) Kausel	2-3	Spherical	Schizogenous	Circular	Strong	Confluent	Scarce
Myrceugenia colchaguensis (Phil.) Navas	2	Rhombohedral	Schizogenous	Arc	Weak	Confluent	Medium
Myrceugenia correifolia (Hook. and Arn.) O.Berg	2-3	Druses	Schizolysigenous	Arc	Weak	Confluent	Medium
Myrceugenia exsucca (DC.) O.Berg	2-3	Druses	Schizogenous	Arc	Weak	Confluent	Abundant
Myrceugenia lanceolata (Juss. ex J. St.-Hil.) Kausel	3	Druses	Schizogenous	Arc	Strong	Not confluent	Abundant

	Mesophyll				Vascular system		
Taxon	P.p. layers	Type of crystals	Type of cavities	Shape	Ad. Phloem partition	Phoem confluence	Amount of ad. phloem
*Myrceugenia leptospermoide*s (DC.) Kausel	2-3	Druses	Schizogenous	Arc	Weak	Confluent	Medium
Myrceugenia obtusa (DC.) O.Berg	2	Druses	Schizolysigenous	Arc	Strong	Confluent	Medium
Myrceugenia ovata (Hook. and Arn.) O.Berg	2-3	Druses	Schizolysigenous	Arc	Strong	Not confluent	Abundant
Myrceugenia ovata var. nanophylla (Burret) L.R. Landrum	2	Druses	Schizogenous	Arc	Strong	Confluent	Abundant
Myrceugenia parvifolia (DC.) Kausel	1	Druses	Schizogenous	Arc	Weak	Confluent	Medium
Myrceugenia pinifolia (F. Phil.) Kausel	2-3	Druses	Schizogenous	Arc	Strong	Confluent	Abundant
Myrceugenia planipes (Hook. and Arn.) O.Berg	2	Spherical	Schizogenous	Arc	Strong	Confluent	Medium
Myrceugenia rufa (Colla) Skottsb. ex Kausel	4	Druses	Schizogenous	Arc	Slight	Not confluent	Scarce
Myrceugenia schulzei Johow	2	Rhombohedral	Schizogenous	Arc	Strong	Not confluent	Medium
Myrcianthes coquimbensis (Barnéoud) Landrum and Grifo	2	Druses	Schizogenous	Circular	Strong	Not confluent	Medium
Myrteola nummularia (Poir.) O.Berg	2-3	Druses	Schizolysigenous	Circular	Absent	Confluent	Scarce

	Mesophyll				Vascular system		
Taxon	P.p. layers	Type of crystals	Type of cavities	Shape	Ad. Phloem partition	Phoem confluence	Amount of ad. phloem
Nothomyrcia fernandeziana (Hook. and Arn.) Kausel	2-3	Druses	Schizogenous	Ellipsoid	Strong	Not confluent	Abundant
Ugni candollei (Barnéoud) O.Berg	2-3	Druses	Schizogenous	Circular	Strong	Not confluent	Medium
Ugni molinae Turcz.	3	Druses	Schizogenous	Arc	Strong	Not confluent	Medium
Ugni selkirkii (Hook. and Arn.) O.Berg	2-3	Rhombohedral	Schizogenous	Arc	Weak	Confluent	Medium
Metrosideros stipularis (Hook. and Arn.) Griseb.	2	Absent	Schizogenous	Circular	Weak	Not confluent	Scarce

P.p: palisade parenchyma

Ad: adaxial

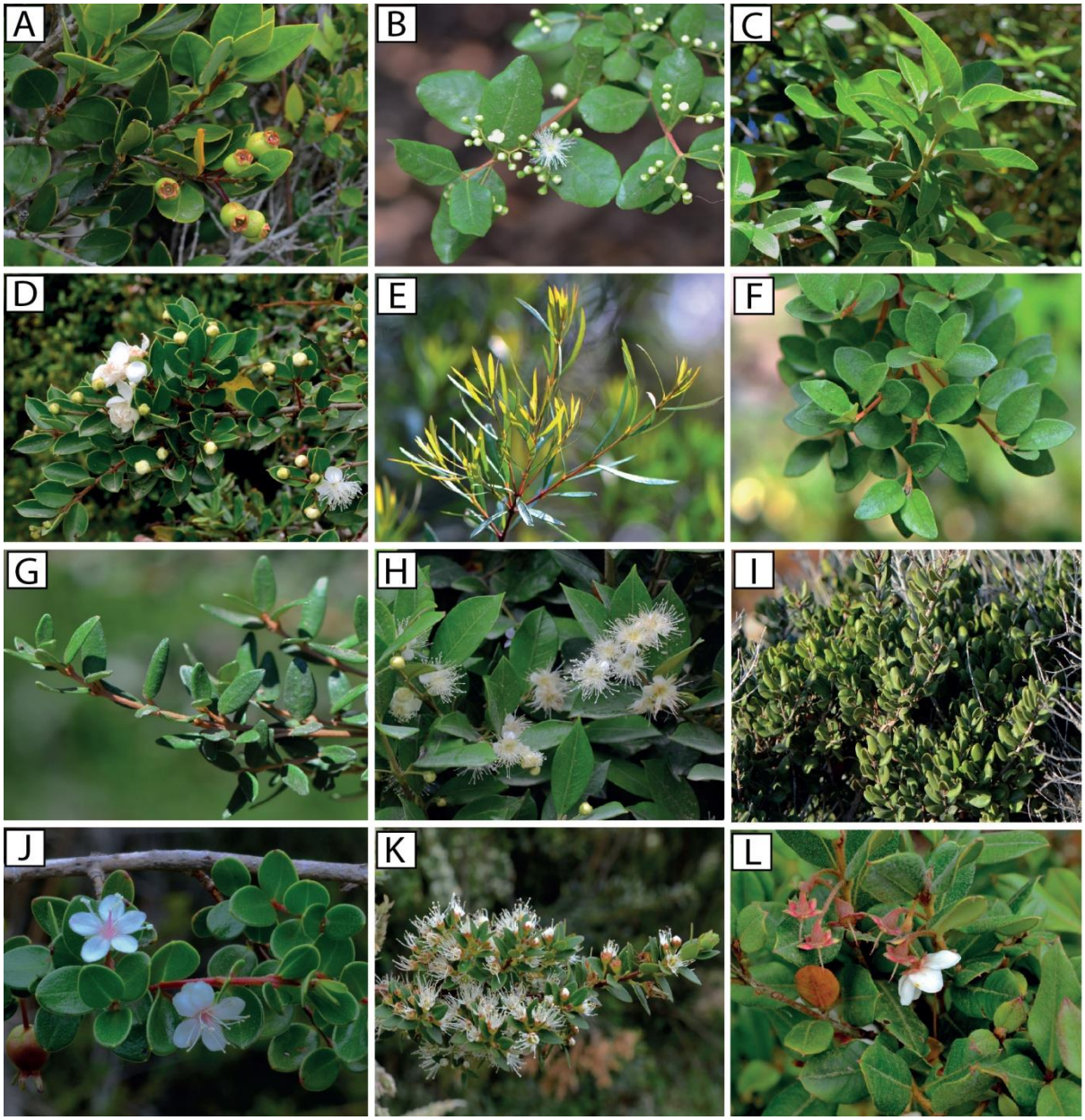


Figure 4.1. Gross morphology of Chilean species of Myrtaceae. A, *Amomyrtus meli*. B, *Blepharocalyx cruckshanksii*. C, *Legrandia concinna*. D, *Luma apiculata*. E, *Myrceugenia lanceolata*. F, *Myrceugenia obtusa*. G, *Myrceugenia rufa*. H, *Myrceugenia planipes*. I, *Myrcianthes coquimbensis*. J, *Myrteola nummularia*. K, *Metrosideros stipularis*. L, *Ugni candollei*.

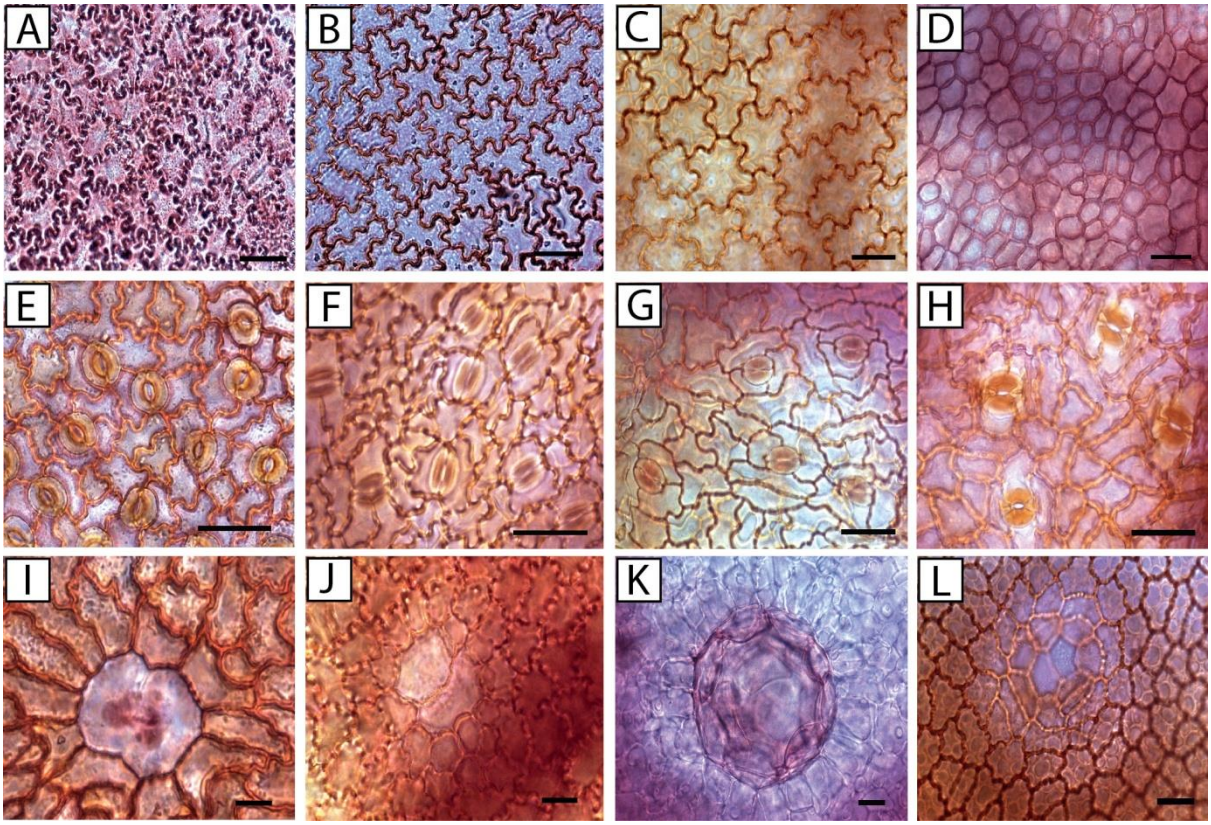


Figure 4.2. Light micrographs (LM) of leaf clearings of Chilean Myrtaceae. A-D, shape of abaxial epidermal anticlinal walls: A, highly sinuous in *Amomyrtus meli*. B, highly sinuous in *Blepharocalyx cruckshanksii*. C, slightly sinuous in *Myrteola nummularia*. D, straight walls in *Myrceugenia correifolia*. E-H, stomatal types: E, anomocytic in *B. cruckshanksii*. F, paracytic in *Luma apiculata*. G, anisocytic in *Ugni candollei*. H, laterocytic in *Myrcianthes coquimbensis*. I-L, secretory cavities: I, cavity showing ca. 10 irregular cells surrounding the two cap cells in *B. cruckshanksii*. J, cavity surrounded by ca. 14 isodiametric cells in *L. concinna*. K, cavity showing eight epithelial cells in *Myrceugenia leptospermoides*. L, cavity surrounded by ca. 7 cells in *Metrosideros stipularis*. Scale bars = 25 µm (A-H), 10 µm (I-L). Stain used: Safranin O.

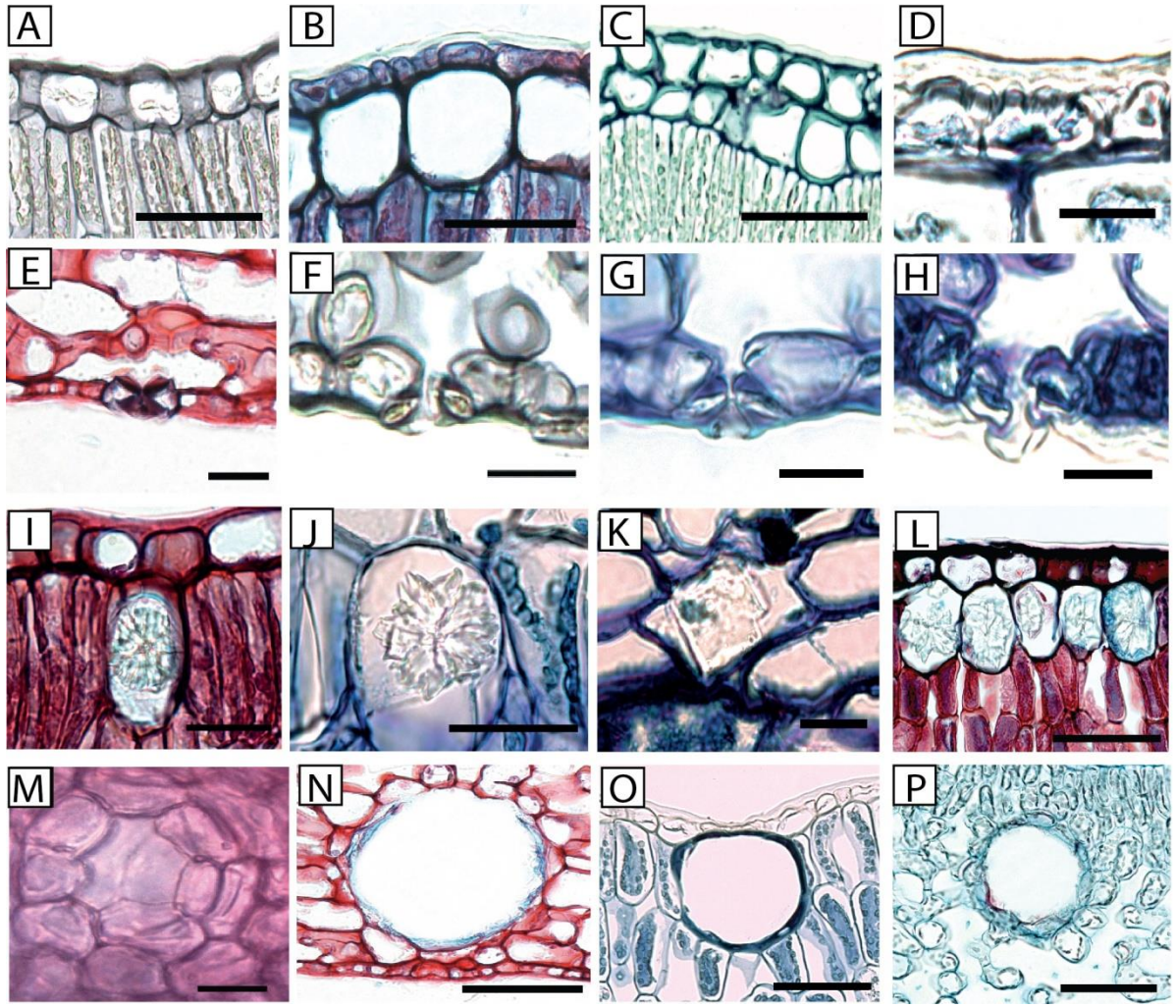


Figure 4.3. Transverse light micrographs (LM) of leaf showing epidermis, stomata and mesophyll elements in Chilean Myrtaceae. A-D, epidermis and cuticle: A, single layered epidermis with thin cuticle in *Luma chequen*. B, thick hypodermis with simple thick cuticle in *Ugni candollei*. C, double epidermis with thick cuticle in *Myrceugenia rufa*. D, single layered epidermis with ornamented cuticle in *Amomyrtus meli*. E-H, transverse view of stomata at equatorial level: E, triangular guard cells with cutinized thickening of outer periclinal walls in *Myrceugenia planipes*. F, ovate guard cells without thickenings in *Luma chequen*. G, heavy cutinized thickenings of outer periclinal walls of guard cells in *Luma apiculata*. H, irregular thickenings in *Ugni selkirkii*. I-L, crystals: I, spherical crystal in *Myrceugenia planipes*. J, druse in *Amomyrtus luma*. K, rhombohedral crystal in *Ugni selkirkii*. L, several grouped druses in *Legrandia concinna*. M-P, secretory cavities: M, early stage of schizogenous secretory cavity showing small and isodiametrical epithelial cells with thin primary walls in *Ugni molinae*. N, schizogenous cavity in spongy parenchyma of *Myrceugenia planipes*. O, schizolysigenous cavity in palisade parenchyma of *Myrteola nummularia*. P, schizolysigenous cavity in the mesophyll of *Luma chequen*. Scale bars = 10 µm (A-D, G, L), 25 µm (E-F, I-K), 50 µm (H, M-P). Stains used: chlorazol black E (A, C, F), TBO (B, D, G, H, J, K, O, P), safranin O - alcian blue (E, I, L, M, N).

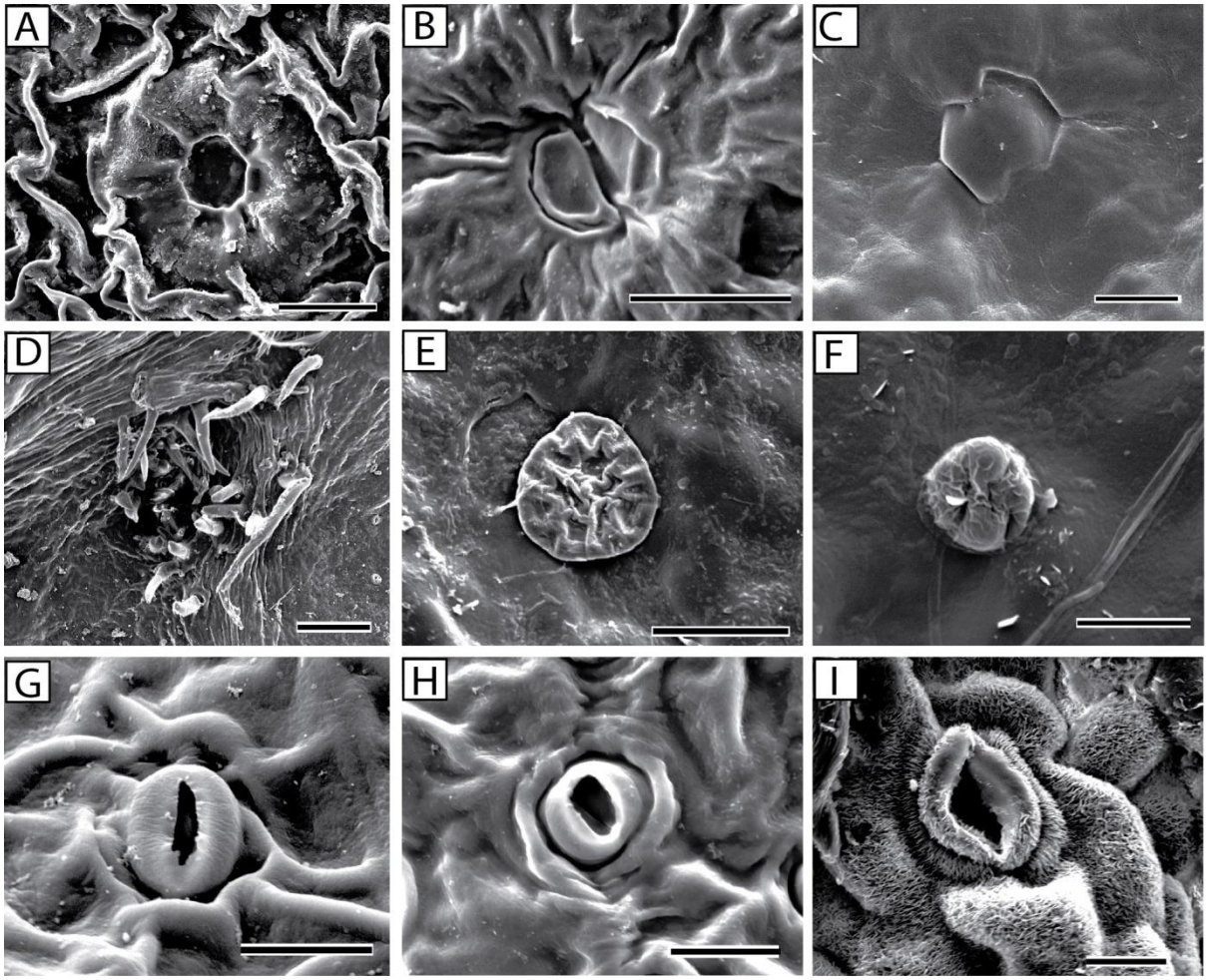


Figure 4.4. Scanning electron micrographs (SEM) of leaf adaxial and abaxial elements of Chilean Myrtaceae. A-C, secretory cavities: A, raised cavity in *Myrceugenia leptospermoides*. B, cavity with two clear overlying cells in *Myrceugenia exsucca*. C, deep secretory cavity with two barely visible overlying cells in *Myrteola nummularia*. D, domatium in *L. concinna* covered with ciliate hairs. E, extrafloral nectary on adaxial surface of *Myrceugenia planipes*. F, extrafloral nectary on adaxial surface of *Metrosideros stipularis*.G-H, stomata with subsidiary cells in G, *Ugni candollei* and H, *Myrceugenia exsucca*. I, stomatal complex surrounded by epidermal cells with hairs and epicuticular waxes in *Myrceugenia colchaguensis*. Scale bars = 25 µm (A-C), 100 µm (D), 10 µm (E-I).

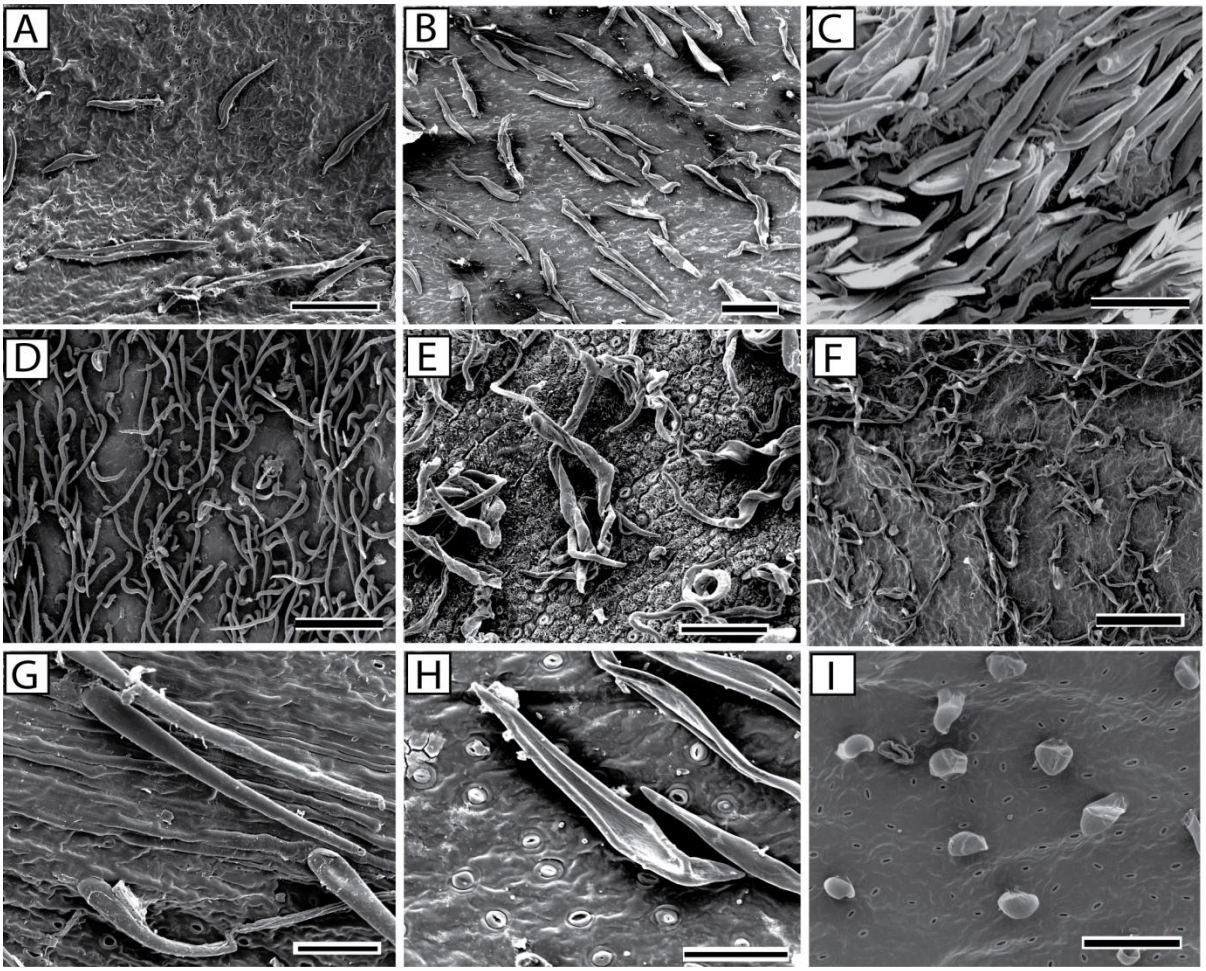


Figure 4.5. Scanning electron micrographs (SEM) of leaf hairs of Chilean Myrtaceae. A-C, abundance of hairs: A, sparsely hairy abaxial surface in *Myrceugenia ovata* var. *ovata*. B, slightly pubescent abaxial surface in *Myrceugenia correifolia*. C, densely hairy abaxial surface in *Myrceugenia rufa*. D-F, distribution of hairs in some pubescent species: D, strongly pubescent leaves with straight hairs in *Myrcianthes coquimbensis*. E, pubescent leaves with twisted hairs in *Myrceugenia schultzei*. F, pubescent leaf with hooked hairs in *Ugni candollei*. G-I, different types of hairs: G, simple hairs in *Amomyrtus luma*. H, symmetrically dibrachiate hairs in *Myrceugenia correifolia*. I, glandular hairs in *Luma chequen*. Scale bars = 50 µm (A-B, G-I), 250 µm (C-D, F), 100 µm (E).

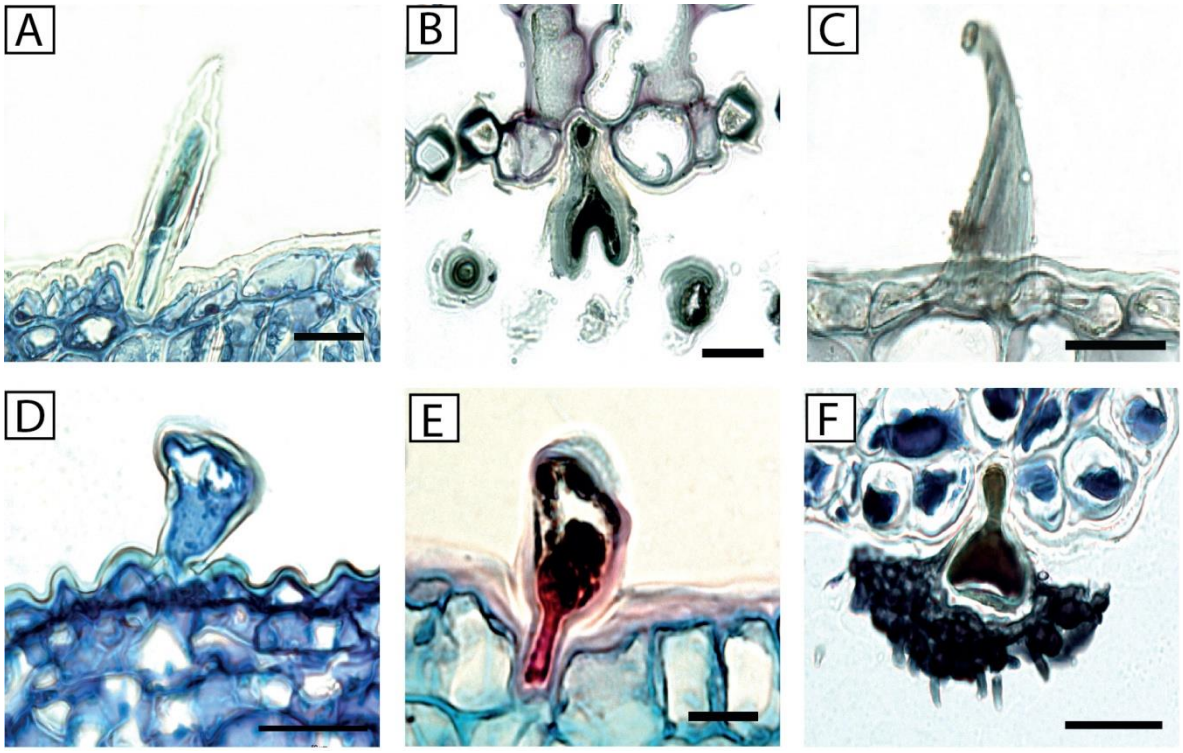


Figure 4.6. Transverse light micrographs (LM) of leaf hairs of Chilean Myrtaceae.A, simple hair in *Amomyrtus luma*. B, symmetrically dibrachiate hair in *Myrceugenia rufa*. C, simple hooked hair in *Ugni candollei*. D-F, glandular hairs: D, *Myrceugenia colchaguensis*. E, *Myrcianthes coquimbensis*. F, *Myrceugenia obtusa* with dark stained secretions around the hair. Scale bars = 40 µm (A), 10 µm (B-F). Stains used: TBO (A, D, F), ruthenium red (B, C), ruthenium red-TBO (E).

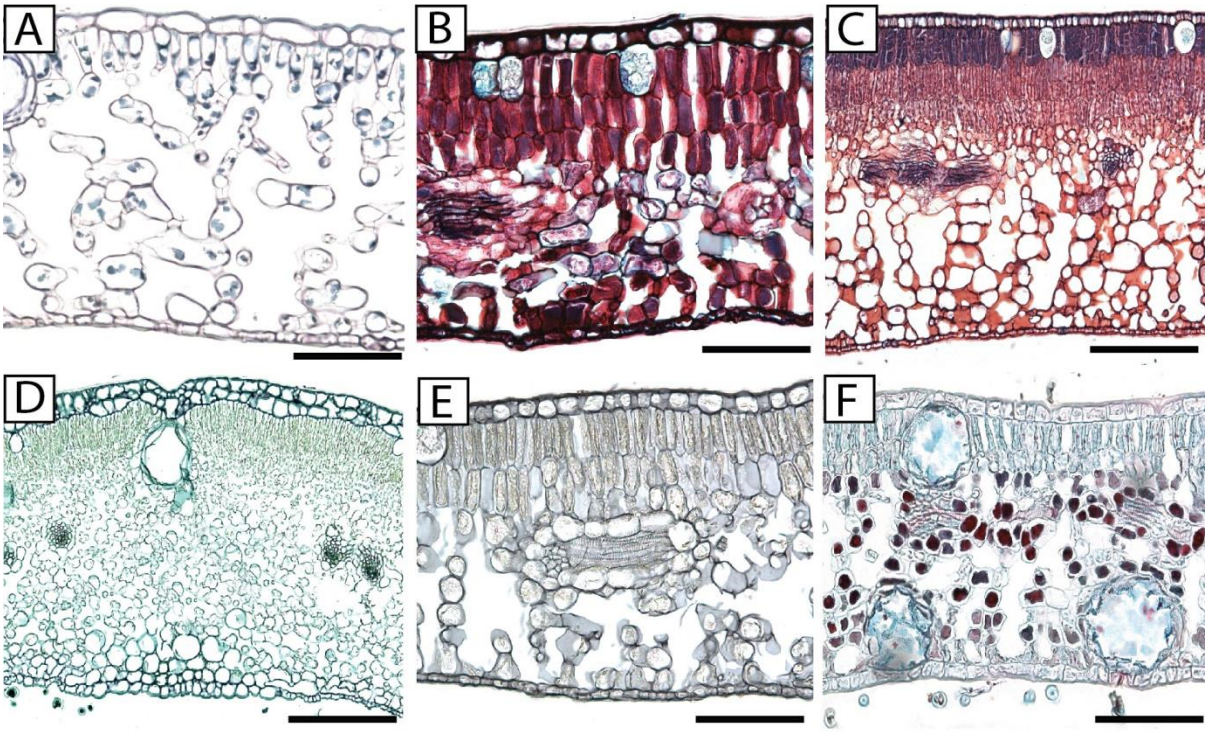


Figure 4.7. Transverse light micrographs (LM) of epidermis and mesophyll of Chilean Myrtaceae. A, single layered epidermis, single layered palisade parenchyma and loose spongy parenchyma in *Myrceugenia parvifolia*. B, single layered epidermis and palisade parenchyma with three layers in *Legrandia concinna*. C, single layered epidermis and compacted palisade parenchyma with three-four layers in *Myrceugenia lanceolata*. D, multiple epidermis and compacted spongy and palisade parenchyma with three layers in *Myrceugenia rufa*. E, single layered epidermis and palisade parenchyma with two layers in *Luma chequen*. F, single layered epidermis, palisade parenchyma with two layers and spongy parenchyma rich in tannins in *Myrcianthes coquimbensis*. Stains used: TBO (A), safranin O - alcian blue (B,C), ruthenium red (D), chlorazol black (E), ruthenium red-TBO (F).

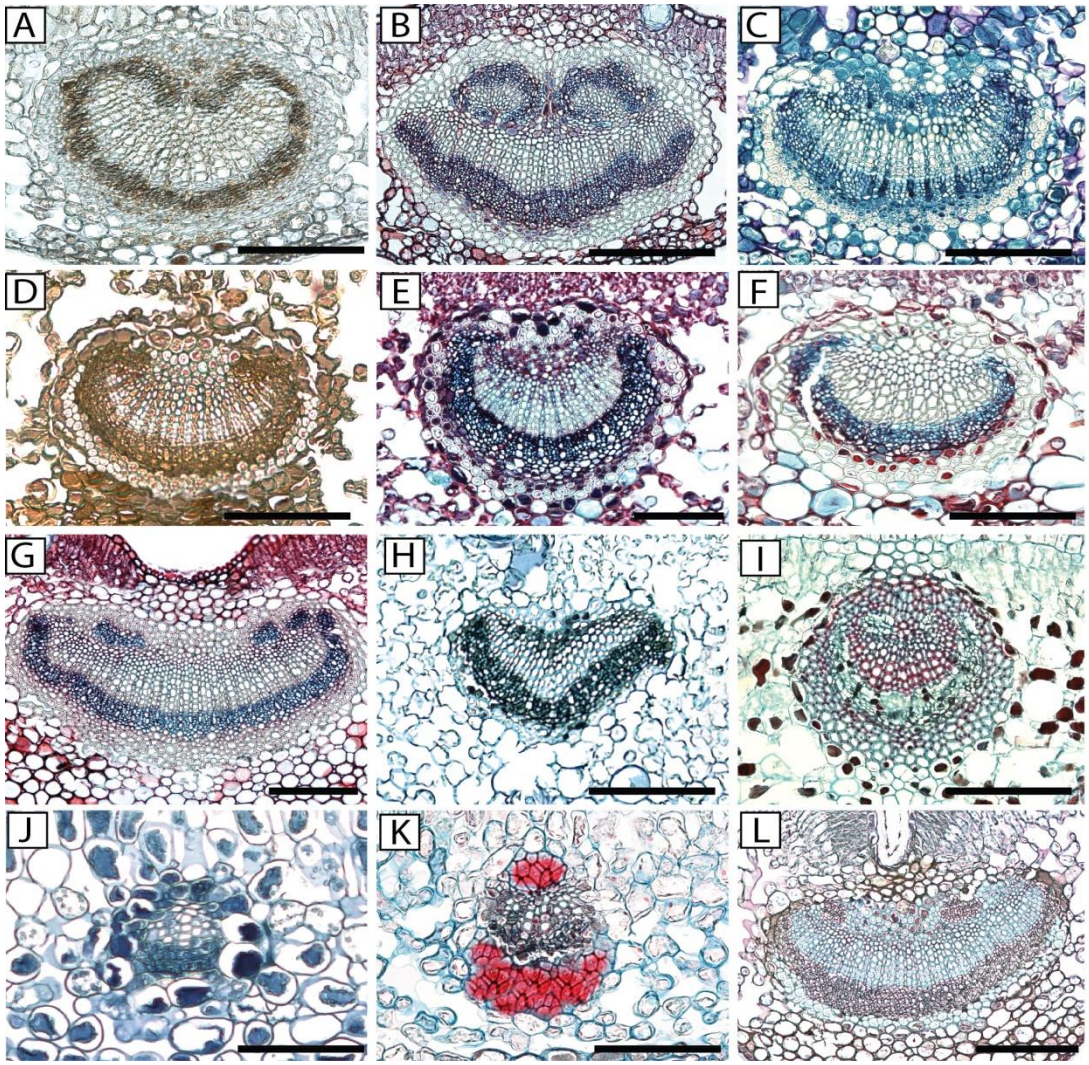
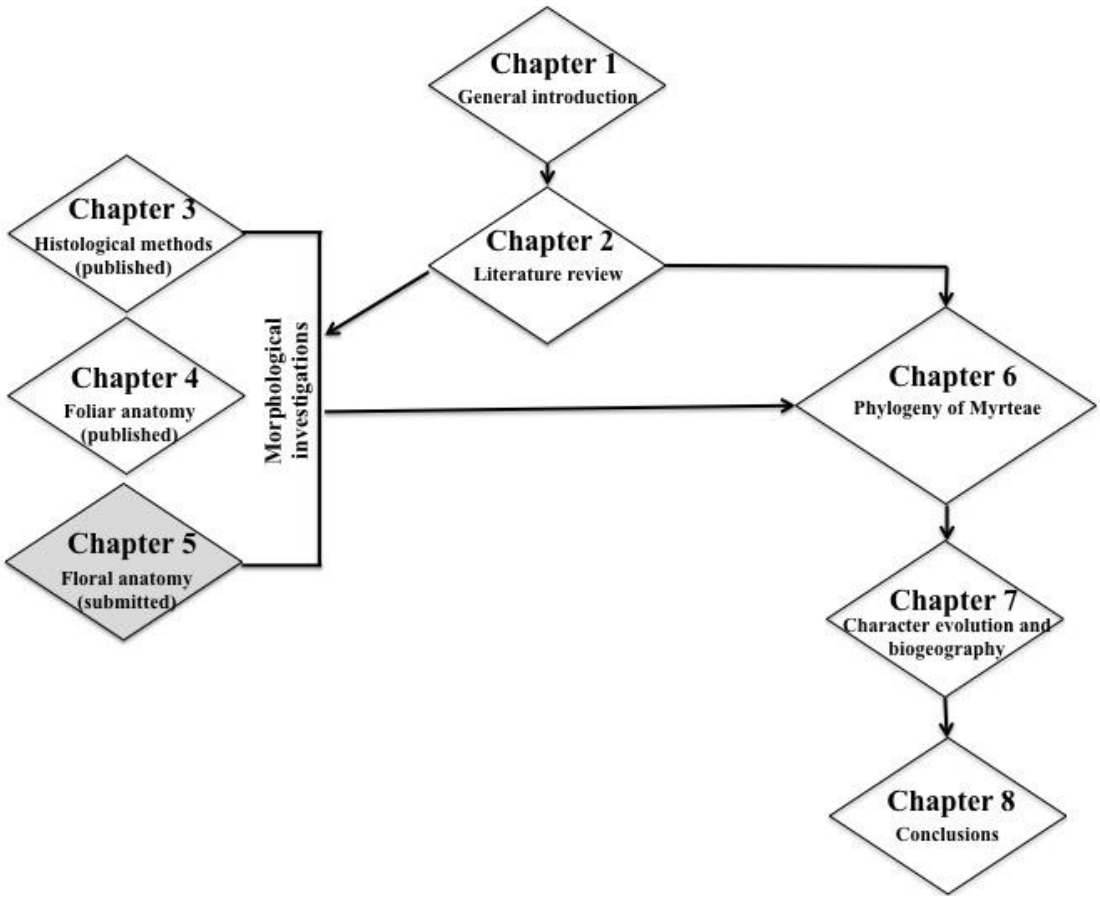


Figure 4.8. Transverse light micrographs (LM) through the leaf vascular system of Chilean Myrtaceae. A, circular vascular system with continuous phloem in *Amomyrtus luma*. B, ellipsoid vascular system with adaxial phloem surrounding two isolated groups of xylem in *Blepharocalyx cruckshanksii*. C, arc-shaped vascular system with abaxial and adaxial confluent phloem in *Legrandia concinna*. D, ellipsoid vascular system with scarce adaxial phloem with strong partition in *Luma apiculata*. E, circular vascular system with strong adaxial phloem partition in *Myrceugenia chrysocarpa*. F arc-shaped vascular system with adaxial and abaxial confluent phloem and strong adaxial partition in *Myrceugenia obtusa*. G, arc-shaped vascular system with strong phloem partition in *Myrceugenia planipes*.H, reduced arc-shaped vascular system in *Myrceugenia rufa*. I, circular midrib with scarce adaxial phloem in *Myrcianthes coquimbensis*. J, reduced circular vascular system with scarce adaxial phloem in *Myrteola nummularia*. K, reduced circular vascular system with scarce adaxial phloem and deeply stained fibres with very thick walls in *Metrosideros stipularis*. L, arc-shaped vascular system with strong adaxial phloem partition in *Ugni molinae*. Scale bars = 100 µm. Stains used: chlorazol black E (A), safranin O - alcian blue (B, E, F, G), TBO (C, J, K), ruthenium red (D, H), ruthenium red - TBO (I, L).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CHAPTER 5: Comparative floral anatomy and micromorphology of the Chilean Myrtaceae

Abstract

A comparative micromorphological and anatomical study was conducted on the species of Chilean Myrtaceae. Micromorphological and anatomical characters related to perianth, hypanthium, androecium and gynoecium where investigated for the first time in the majority of the species. The majority of species have tetramerous flowers, glandular sepals and petals, numerous (100+) semi-curved stamens with secretory cavities and abundant tannins in the mesophyll of petals. Calcium oxalate crystals were observed in the anthers of nearly all the species. Most of the species have an indefinite number of receptacular bundles, while a few species have consistently eight (*Amomyrtus* spp., *Legrandia concinna*, *Myrcianthes coquimbensis*) or five (*Myrceugenia exsucca*, *Myrceugenia planipes*). *Amomyrtus* spp., *Myrcianthes coquimbensis*, *Myrteola nummularia* and *Ugni molinae* have a very conspicuous nectariferous region in the superior portion of the hypanthium, characterized by dense cytoplasm and large nuclei. Two types of placentation (ovules along a non-peltate placenta, ovules along a protruding placenta) were observed across the species. Ovules have two integuments in all the species, except for *Metrosideros stipularis* (tribe Metrosidereae), which has ovules with one integument. Results of this investigation provide an insight into the reproductive biology of the species of Chilean Myrtaceae. Characters described here might be potentially useful for phylogenetic analyses and for ecological studies of these species. This is the first anatomical reproductive survey conducted on all representatives of a family of plants occurring in the Chilean forests.

5.1 Introduction

The study of floral anatomy is critically important to understanding the reproductive mechanisms (Rudall et al., 2002) and might provide an important source of taxonomic information (Schmid, 1972). Little is known regarding the floral anatomy and micromorphology of Myrtaceae, with only few studies comprehensively investigating the reproductive anatomy of some species (e.g., Schmid, 1972; Belsham and Orlovich, 2003; Ciccarelli et al., 2008; Pimentel et al., 2014). Schmid (1972) described in detail the floral anatomy of several species of *Eugenia* and *Syzygium* and established a number of characters

to differentiate the two genera, including number of receptacular bundles and type of vascular supply to the ovules. Pimentel et al. (2014) described the floral anatomy of several Myrteae species and reported the number of integuments, presence of sclereids for the first time and interpreted the origin of the placenta in Myrteae. Studies on the floral micromorphology (SEM) of Chilean Myrtaceae are rare, only known for *Luma apiculata*, *Ugni molinae* (Belsham and Orlovich, 2002, 2003) and some species of *Myrceugenia* (Landrum, 1981b). Additional, more recent papers by these authors have not been cited as the work is on other tribes in Myrtaceae and not Myrteae. Floral anatomy in Chilean Myrtaceae has only been studied in *Myrceugenia rufa* (Retamales et al., 2014a).

Flowers in the family Myrtaceae are mostly actinomorphic, 4-5 merous and bisexual (Fig. 5.1). Sepals and petals are usually free, petals are imbricated and caducous, stamens are multiseriate, numerous and free or in clusters, anthers are dorsifixed and versatile in most species, mainly dehiscing by slits (Schmid, 1972; Wilson, 2011). Some species have basifixed anthers (e.g., some *Eucalyptus* species) (Wilson, 2011). The ovary is partially inferior to fully inferior with 1-5 carpels, placentation parietal, axile or basal and there is usually a hypanthium with nectaries (Johnson and Briggs, 1984; Conti et al., 1997). The number of ovules ranges from two to many and ovules are mostly anatropous, hemitropous or campylotropous (Wilson, 2011). Inflorescences in the family are mostly determinate and variable in type (e.g., dichasia, triads, single flowers, panicles, thyrsoids, botryioids) (Johnson and Briggs, 1984; Wilson, 2011).

In terms of gross morphology, flowers of Myrtaceae have been reported as uniform and similar (Pimentel et al., 2014). It is necessary to confirm whether anatomical and micromorphological characters described for other Myrtaceae are also found in Chilean Myrtaceae and whether these characters are phylogenetically informative. A comprehensive comparative study of the floral anatomy of all the genera of Chilean Myrtaceae may provide insightful information regarding taxonomy, phylogenetics and reproductive biology of the species. In this investigation, the anatomy and micromorphology of all the genera of Chilean Myrtaceae was described for the first time. Herein, we will not focus on those gross morphological and micromorphological characters studied in detail in taxonomic revisions (Landrum, 1981b, 1986, 1988a, 1988b; Landrum and Stevenson, 1986; Landrum and Grifo, 1988). Information gathered from taxonomic revisions will be incorporated into subsequent phylogenetic analyses (Chapter 6) along with anatomical and micromorphological data generated in this investigation.

5.2 Material and methods

5.2.1 Material examined

The micromorphology and anatomy of all 26 species of Chilean Myrtaceae was examined. Flower anatomy of few species was described based on fragments due to insufficient quality of some flower buds and open flowers obtained from herbarium specimens. Micrographs were not obtained for those species with insufficient quality. Flowers at different maturation states (flower bud, pre-anthesis, anthesis) were randomly sampled from typical healthy individuals. Fresh flowers were fixed in formalin-acetic acid-alcohol (FAA) for 12 h and subsequently stored in 70% Ethanol. Herbarium specimens were rehydrated in boiling water for 10 min to recover shape before being fixed in FAA (Haron and Moore, 1996). A detailed study of pollen was not carried out. Herbarium accessions are currently deposited in the Forestry Sciences Herbarium, University of Chile (EIF), with duplicates housed in the Queensland Herbarium, Brisbane, Australia (BRI). Details of species studied and vouchers are presented in Appendix 1.

5.2.2 Scanning electron microscopy (SEM)

Flower material fixed in FAA was dehydrated using a graded ethanol series and then critical point dried (Anderson, 1951) using Ethanol in an Autosamdri-815 automatic critical point drier (Tousimis, Rockville, USA). Floral samples were mounted on stubs with self-adhesive double-sided carbon discs and sputter-coated with gold palladium for 175 sec using a Leica EM SCD005 Gold Coater (Leica Microsystems, Macquarie Park, NSW, Australia). Examination and documentation of images was undertaken using a FEI Quanta 200 SEM/ESEM (FEI, Oregon, USA) operated at 10kV at CARF (Central Analytical Research Facility, QUT).

5.2.3 Light microscopy (LM)

FAA-fixed material was dehydrated using ethanol series and subsequently embedded in paraffin wax (Johansen, 1940; Ruzin, 1999). Transverse and longitudinal sections of flowers were cut using a Leica RM2245 rotary microtome (Leica Microsystems, Macquarie Park, NSW, Australia) at 5µm. Sections were stained Ruthenium red (0.05% aqueous solution), Toluidine blue (TBO) (0.1% aqueous solution), Safranin O (1% alcoholic solution) and alcian blue, alone or combined according to standard staining protocols (Ruzin, 1999; Retamales and Scharaschkin, 2014). A staining protocol developed in Chapter 3 of this thesis was used

to identify secondary compounds in flowers (e.g., tannins, polyphenols, polysaccharides). Staining and processing of paraffin sections followed the histological protocols detailed in Retamales et al. 2015 (Chapter 4). The chemical nature of intracellular crystals was tested by adding 1µl of acetic acid (5%) and 1µl of hydrochloric acid (9%) to sections (Maclean and Ivimey-Cook, 1952). Sections were mounted using DPX (Sigma-Aldrich Co., St. Louis, Missouri, USA).

5.2.4 Taxonomy and terminology

The terminology for describing flower anatomy and micromorphology used here was based on previous descriptions of Schmid (1972), Landrum (1981a, 1988a), Belsham and Orlovich (2003), Ciccarelli et al. (2008) and Pimentel et al. (2014). Other general references consulted for anatomical terminology include Gifford and Foster (1989), Dickison (2000), Evert (2006) and Pole (2010). Terminology for placentation was directly taken from Lucas et al. (2007). Different types of stomata were not determined in this study, since SEM images did not provide clear distinction between them and clearings of petals were not conducted. It was not possible to examine the stigma of most of the species in detail, since cells were shrunken or damaged.

5.3 Results

This section presents the results on the floral micromorphology and anatomy of the Chilean Myrtaceae. Descriptions will be grouped by character including both micromorphological and anatomical observations for all the studied species. The first part of the results will be an overall description of flower buds and flowers, followed by the description of each character, namely indumentum, perianth, secretory cavities, hypanthium, androecium and gynoecium. The abbreviation spp. was used when many species in a genus had the same character state. Results of this investigation are summarized in Table 5.1.

5.3.1 General overview of flower buds and flowers

The majority of species possess tetramerous flowers, but some species have pentamerous flowers, namely *Amomyrtus* spp., *Metrosideros stipularis*, *Myrcianthes coquimbensis* and *Ugni* spp. *Myrteola nummularia* has both tetramerous and pentamerous flowers, while some samples of *Ugni molinae* have both tetramerous and trimerous flowers. In flower buds, sepals enclose the petals leaving swellings at the top of the buds. Sepals are free and glandular (with abundant secretory cavities) in most of the species. A layer of hairs covers the sepals and

hypanthium in the majority of species (Fig. 5.2). Petals are mostly white, pinkish in some species such as *Ugni molinae* and *Ugni candollei*. Bracteoles are observed in all species, which are either persistent or caducous after anthesis. Bracteoles are linear, lanceolate or narrowly elliptic in most of the species, while foliaceous in *Legrandia concinna*, *Myrteola nummularia* and *Myrceugenia obtusa*. The stamens are numerous (100+) in the majority of the species, except for *Myrteola nummularia* (7-18 stamens) and *Metrosideros stipularis* (15-20 stamens). The stamens are erect in species such as *Myrcianthes coquimbensis*, *Myrteola nummularia* and *Amomyrtus* spp., while semi-curved stamens are observed in *Myrceugenia* spp. The ovary is inferior and has 2-4 locules and 2-many ovules per locule in all the species.

5.3.2 Indumentum

All species have hairs on different parts of flowers, mainly on the abaxial and adaxial surfaces of sepals, margins of sepals and petals and hypanthium (Figs 5.3A, 5.3B, 5.3C). *Myrteola nummularia* possesses glabrous flowers or nearly so, with hairs present only at the base of the internal surface of sepals. The flowers become glabrescent with age in most of the species. The majority of species have a sparse layer of hairs on the adaxial surface of sepals or are essentially glabrous, while other have a dense layer of hairs (e.g., *Myrceugenia* spp., *Myrcianthes coquimbensis*, *Ugni candollei*) (Fig. 5.3C). *Blepharocalyx cruckshanksii* is essentially glabrous but possesses a dense layer of hairs only on the abaxial surface of sepals in some species (Fig. 5.3A). Some species do not possess conspicuous hairs on the adaxial surface of sepals (e.g., *Myrceugenia ovata* var. *ovata*, *Myrceugenia parvifolia*, *Myrceugenia planipes*). Most of the species have glabrous petals. A very dense layer of hairs on the hypanthium was observed in many species (e.g., *Myrceugenia colchaguensis*, *Myrceugenia rufa*) (Figs 5.2C, 5.2G, 5.2H). Species in the genus *Luma* (*L. apiculata* and *L. chequen*), as well as *Blepharocalyx cruckshanksii* and *Metrosideros stipularis* do not have hairs on the hypanthium (Fig 5.2B). Hairs on sepals, petals and hypanthium are either simple (*Amomyrtus* spp., *Luma* spp., *Myrcianthes coquimbensis*, *Ugni* spp.) or dibrachiate (*Myrceugenia* spp., *Ugni candollei*) (Fig. 5.2C) (Table 5.1).

5.3.3 Perianth

The majority of the species are tetramerous (e.g., *Blepharocalyx cruckshanksii*, *Legrandia concinna*, *Luma* spp., *Myrceugenia* spp., *Nothomyrcia fernandeziana*), but some are pentamerous (*Amomyrtus* spp., *Myrcianthes coquimbensis*, *Metrosideros stipularis*) (Fig. 5.4). Some individuals of *Myrteola nummularia* have pentamerous and tetramerous flowers

in the same individual. The three species of *Ugni* are mainly pentamerous but some flowers in the same individual might have tetramerous and more rarely trimerous flowers. Sepals have a thin cuticle (2-3 μm) in most of the species. Stomata are observed on the abaxial surface of sepals, mainly observable in glabrous-sparsely hairy species (e.g., *Blepharocalyx cruckshanksii*, *Luma* spp.). The epidermis of sepals is single layered with isodiametric cells and slightly undulated-straight anticlinal walls (Fig. 5.4D). The mesophyll of sepals has generally many cell layers and cells have thick cell walls (Figs 5.5A, 5.5B). In some species, there are several cell layers in the mesophyll of sepals (e.g., *Ugni candollei*) (Fig. 5.5B). Cells in the mesophyll of sepals are generally rounded, with regular air spaces between cells. Petals generally do not have an obvious cuticle or have a thin and sparse wax deposition (1 μm). Stomata are observed on the abaxial surface of petals. The epidermis of petals is single layered with isodiametric cells and slightly undulated-straight anticlinal walls. Cellular contents of epidermal cells of petals are most probably tannins, based on the staining reaction with TBO-Ruthenium red (5.5A). The mesophyll of petals has few cell layers and cells have thin cell walls (Fig. 5.5B). In most species, cells in the mesophyll of petals are probably rich in tannins, based on the staining reaction.

5.3.4 Secretory cavities

Secretory cavities are numerous and generally located in contact with the epidermis of the hypanthium, sepals and/or petals (Fig. 5.4, 5.5C). The majority of species possess secretory cavities in both sepals and petals (Fig. 5.4). A few species possess secretory cavities in the sepals but not in the petals (e.g., *Metrosideros stipularis*, *Myrcianthes coquimbensis*, *Ugni candollei*). The presence of secretory cavities in the hypanthium was difficult to assess for species with limited floral material. This character was not included in the subsequent phylogenetic analyses (Chapter 6). In SEM surface view, secretory cavities are observed as swellings (glands) on the hypanthium and sepals in most of the species (Figs 5.3E, 5.3F). Secretory cavities in anthers were observed in some species. These are mainly located in the connective tissue of the anthers. In younger buds, secretory cavities are initially formed by separation of epithelial cells, which indicates that cavities are not lysigenous. Species have mainly schizogenous secretory cavities, but some species possess schizolysigenous cavities (e.g., *Blepharocalyx cruckshanksii*, *Myrteola nummularia*). Histochemical reactions with Sudan IV indicate the presence of lipophilic substances, possibly terpenes and sesquiterpenes in most cavities.

5.3.5 Hypanthium

The hypanthium epidermis is single layered, composed of enlarged cells with thin cell walls and abundant stomata. The hypanthium has a peripherical region composed of large parenchymatic cells with scarce cellular content and an internal nectariferous region composed of small and tightly packed cells with abundant cell content (Fig. 5.5D). Cells of the nectariferous region have dense cytoplasm, large nuclei and thin cell walls. The nectariferous region extends from the middle to about the top of the hypanthium. The nectariferous region is very conspicuous in *Amomyrtus* spp., *Myrceugenia exsucca*, *Myrcianthes coquimbensis*, *Myrteola nummularia* and *Ugni molinae*. The other species of Chilean Myrtaceae do not show a differentiated nectariferous region in the hypanthium.

5.3.6 Androecium

Stamens are numerous in most of the species (100+ stamens) (Fig. 5.6A), except for *Myrteola nummularia* and *Metrosideros stipularis*, which have fewer stamens (7-18; 15-20 respectively) (Fig. 5.4). The internal tissue of the filament is formed by parenchymatous tissue with secretory cavities and a concentric vascular bundle. The epidermis of the filament is composed of rectangular or isodiametric cells with thin cell walls and cellular content is stained positively with TBO (light green), which indicates the presence of polyphenols. The anthers have two thecae, each with two microsporangia (pollen sacs). The connective tissue is composed of parenchymatous cells and a vascular bundle in the middle with a concentric ring of phloem around the xylem (Fig. 5.6B). Druses are observed in the anthers of some species. The nature of crystals was determined as calcium oxalate (CaOx) after dissolution with acetic and hydrochloric acid. The connective tissue has idioblasts that contain calcium oxalate druses in some species (e.g., *Myrceugenia exsucca*, *Ugni* spp.). The structure of the anther from the outside to the inside is as follows: a thin epidermis, a thick endothecium, a diffuse middle layer and a generally detached tapetum formed by a thin layer of secretory cells. In mature anthers, the endothecium has strips of secondary thickenings on the outer periclinal walls (Fig. 5.6C). Triangular pollen grains are observed in the pollen sacs. The anatomy of the androecium is similar between all the species of Chilean Myrtaceae, with the exception of *Ugni* spp., where the connective tissue is developed as an extended glandular tip. Besides, the stamens are clustered and closely attached to the style in the *Ugni* species.

5.3.7 Gynoecium

The gynoecium in all Chilean Myrteae is clearly differentiated into stigma, style and ovary. The style possesses a papillose single layered epidermis rich in tannins in most of the species. The first two subepidermal layers are similar to the epidermis in shape and size and also contain abundant tannins, while inner cell layers correspond to loose parenchyma that did not show staining reaction (Fig. 5.6F). The central region of the style possesses between two and six vascular bundles and a transmitting tissue mainly formed by phloem with dense cytoplasm in all species (Fig. 5.6F). The ovary, which is inferior in all species, is mainly formed by parenchymatous tissue (Fig. 5.6D). Parenchymatous cells of the ovary are rounded, compacted and have thin cell walls in all species. The number of receptacular vascular bundles varies depending upon species, with the majority of taxa having indefinite number and others eight receptacular bundles (e.g., *Myrcianthes coquimbensis*, *Amomyrtus* spp., *Legrandia concinna*) or five (e.g., *Myrceugenia planipes, Myrceugenia exsucca*) (Fig. 5.6D). The number of carpels ranges from two (*Myrcianthes coquimbensis*, *Luma* spp.) to three and four (*Ugni* spp.). Nearly all the species possess ovaries with two-three carpels. The number of ovules per locule ranges from 4-6 in *Amomyrtus meli* to 8-32 in *Ugni candollei*. The number of ovules per locule ranges from six to 20 in the majority of species (e.g., *Metrosideros stipularis*, *Blepharocalyx cruckshanksii*, *Legrandia concinna*, *Myrceugenia* spp.). Two types of placentation have been identified across the species: ovules in one or more rows along a non-peltate placenta (*Luma* spp., *Myrceugenia* spp.) and ovules in one or more rows along a protruding placenta (*Amomyrtus* spp., *Legrandia concinna*, *Myrcianthes coquimbensis, Myrteola nummularia, Ugni* spp.). Ovules have two integuments in all species, except for *Metrosideros stipularis*, which have one integument. The nucellus is formed by thin-walled compacted and vacuolated cells (Fig. 5.6E).

5.4 Discussion

Anatomical and micromorphological characters examined here partially agree with previous observations in the family Myrtaceae. The taxonomic, ecological and phylogenetic value of flower anatomy has been widely recognised for Myrtaceae (Schmid, 1972; Pimentel et al., 2014; Vasconcelos et al., 2015). It is here confirmed that floral anatomy and micromorphology is potentially useful as phylogenetic information, since characters vary among genera and species. Gross morphology of flowers largely agrees with the taxonomic revisions of Kausel (1947), Landrum (1981, 1988a, 1988b) and Wilson (2011) in terms of

number of perianth elements, stamens, carpels and ovules. The stamen position among Chilean species of Myrtaceae agrees with the observations of Vasconcelos et al. (2015), who reported erect stamens in species of the "*Eugenia* group", "*Myrteola* group" and "*Pimenta* group" (Lucas et al., 2007) (e.g., *Myrcianthes coquimbensis*, *Myrteola nummularia*, *Amomyrtus* spp.). Species of *Myrceugenia* were confirmed with a semi-curved stamen pattern (Vasconcelos et al. 2015). The two types of hairs found in flowers and flower buds (simple and dibrachiate) have been previously reported in Chilean species of Myrtaceae (Landrum, 1988). A third type of hair (glandular) found in leaves of Chilean Myrtaceae (Retamales et al., 2015) was not observed in this study. Distribution on hairs on sepals, petals and hypanthium observed in this investigation largely agrees with descriptive studies on Chilean Myrtaceae (Reiche, 1897; Kausel, 1942; Landrum, 1988). The cuticle present in sepals but inexistent (or extremely thin) in petals, has been also reported in previous studies on flower anatomy (Rosa and Scatena, 2007; Nunes et al., 2013). An internal nectariferous region can be easily recongnised in many genera (e.g., *Amomyrtus*, *Myrteola*, *Myrcianthes*, *Ugni*). This character has been described here for the first time for Myrtaceae. The anatomy and micromorphology of the gynoecium and androecium largely agrees with the descriptions made by Pimentel et al. (2014) and Vasconcelos et al. (2015) respectively. The number of receptacular bundles in the ovary of *Eugenia* and *Syzygium* species was studied by Schmid (1972) and indicated as an informative character to delimitate species and the two genera. Further studies of this character have reported that species of the tribe Myrteae have either a definite number (eight) or an indefinite number of receptacular bundles. However, in this study we have identified species having consistently five receptacular vascular bundles (*Myrceugenia planipes*, *Myrceugenia exsucca*). Histochemical observations in flowers suggested the presence of tannins and sesquiterpenes in petals, similar to those found by Schmid (1972) and Ciccarelli et al. (2008) in flowers of *Eugenia*, *Syzygium* and *Myrtus communis*. In this study, tannins and pigments are mainly found in vacuoles and phloem companion cells, while the origin and storage of these compounds have not been specified in previous studies on Myrtaceae. Chemical compounds produced by secretory cavities in flowers (terpenes and/or sesquiterpenes) are similar to those reported in leaves of Myrtaceae (Stefanello et al., 2014; Retamales et al., 2014b; Godinho et al., 2014). Abundant secretory cavities observed in SEM view in some species (e.g., *Luma chequen*, *Myrceugenia obtusa*), agree with gross morphological observations of these species, which have been reported as glandular species (Kausel, 1947; Landrum, 1988). Secretory cavities in anthers have been reported in species of Myrtaceae only by Schmid (1972). The abundance of chemical

compounds in different parts of the flower has been cited as an important character for the reproductive biology and ecology of the species, including pollination, herbivory and defence against pathogens (Rudall et al., 2002; Litt and Stevenson, 2003; Rosa and Scatena, 2007).

5.5 Conclusion

Examination of the floral anatomy and micromorphology of the species of Chilean Myrtaceae highlighted a number of characters that might provide insightful information for taxonomic and phylogenetic studies. Characters described here might form the basis for future ecological studies focused on the species of the Chilean forest. Some characters described here include the presence of crystals in anthers, abundance of tannins in style and petals, number of receptacular bundles and presence of a conspicuous nectariferous region in the hypanthium. This is the first anatomical and micromorphological description for the majority of the species examined here.

Table 5.1. Floral anatomical and micromorphological characters of Chilean Myrtaceae. Please see Appendix 1 for taxonomic authorities.

Taxon	Pubescence on sepals	Pubescence on petals	Type of hairs on petals and sepals	Tannin content in petals	Cavities in anthers	Crystals in anthers	Nectariferous region	Receptacular bundles	Cavities in hypanthium	Cavities in sepals	Cavities in petals
Amomyrtus luma	Dense	Glabrous	Simple	Abundant	Present	Present	Conspicuous	Eight	Present	Present	Present
Amomyrtus meli	Glabrous	Glabrous	Simple	Abundant	Present	Present	Conspicuous	Eight	Absent	Present	Present
Blepharocalyx cruckshanksii	Glabrous	Glabrous	Simple	Abundant	Present	Absent	Inconspicuous	Indefinite	Absent	Present	Present
Legrandia concinna	Sparse	Glabrous	Simple	Scarce	Present	Present	Inconspicuous	Eight	-	Present	Present
Luma apiculata	Sparse	Glabrous	Simple	Abundant	Present	Absent	Inconspicuous	Indefinite	Present	Present	Present
Luma chequen	Glabrous	Glabrous	Simple	Abundant	Present	Absent	Inconspicuous	Indefinite	-	Present	Present
Myrceugenia chrysocarpa	Sparse	Glabrous	Dibrachiate	Abundant	Present	Absent	Inconspicuous	Indefinite		Present	Present
Myrceugenia colchaguensis	Dense	Dense	Dibrachiate	Abundant	Present	Present	Inconspicuous	Indefinite	Present	Present	Present
Myrceugenia correifolia	Dense	Dense	Dibrachiate	Abundant	Present	Absent	Inconspicuous	Indefinite		Present	Present
Myrceugenia exsucca	Dense	Dense	Dibrachiate	Abundant	Present	Absent	Conspicuous	Five	Present	Present	Present
Myrceugenia lanceolata	Sparse	Glabrous	Dibrachiate	Abundant	Present	Present	Inconspicuous	Indefinite	Present	Present	Present
Myrceugenia leptospermoides	Glabrous	Glabrous	Dibrachiate	Abundant	Present	Present	Inconspicuous	Indefinite	Present	Present	Present
Myrceugenia obtusa	Sparse	Glabrous	Dibrachiate	Abundant	Present	Present	Inconspicuous	Indefinite	Present	Present	Present
Myrceugenia ovata	Glabrous	Glabrous	Dibrachiate	Abundant	Present	Present	Inconspicuous	Indefinite	Present	Present	Present

Taxon	Pubescence on sepals	Pubescence on petals	Type of hairs on petals and sepals	Tannin content in petals	Cavities in anthers	Crystals in anthers	Nectariferous region	Receptacular bundles	Cavities in hypanthium	Cavities in sepals	Cavities in petals
M. ovata var. nanophylla	Glabrous	Glabrous	Dibrachiate	Abundant	Present	Present	Inconspicuous	Indefinite	-	Present	Present
Myrceugenia parvifolia	Glabrous	Glabrous	Dibrachiate	Abundant	Absent	Present	Inconspicuous	Indefinite	Present	Present	Present
Myrceugenia pinifolia	Sparse	Glabrous	Dibrachiate	Abundant	Present	Present	Inconspicuous	Indefinite	-	Present	Present
Myrceugenia planipes	Glabrous	Glabrous	Dibrachiate	Abundant	Present	Present	Inconspicuous	Five	Present	Present	Present
Myrceugenia rufa	Dense	Dense	Dibrachiate	Abundant	Present	Present	Inconspicuous	Indefinite	Present	Present	Present
Myrceugenia schulzei	Dense	Dense	Dibrachiate	Abundant	Present	Present	Inconspicuous	Indefinite	-	Present	Present
Myrcianthes coquimbensis	Dense	Glabrous	Dibrachiate	Abundant	Present	Present	Conspicuous	Eight	Present	Present	Absent
Myrteola nummularia	Glabrous	Glabrous	Simple	Abundant	Present	Absent	Conspicuous	Indefinite	Present	Present	Present
Nothomyrcia fernandeziana	Sparse	Glabrous	Simple	Abundant	Present	Absent	Inconspicuous	Indefinite	-	Present	Present
Ugni candollei	Dense	Dense	Dibrachiate	Abundant	Present	Present	Inconspicuous	Indefinite	Present	Present	Absent
Ugni molinae	Sparse	Glabrous	Simple	Abundant	Present	Present	Conspicuous	Indefinite	Present	Present	Present
Ugni selkirkii	Dense	Dense	Simple	Abundant	Absent	Present	Inconspicuous	Indefinite	Absent	Present	Absent
Metrosideros stipularis	Sparse	Glabrous	Simple	Abundant	Present	Present	Inconspicuous	Indefinite	Absent	Present	Absent

- Not possible to retrieve information for that character (e.g., specimen of poor quality).

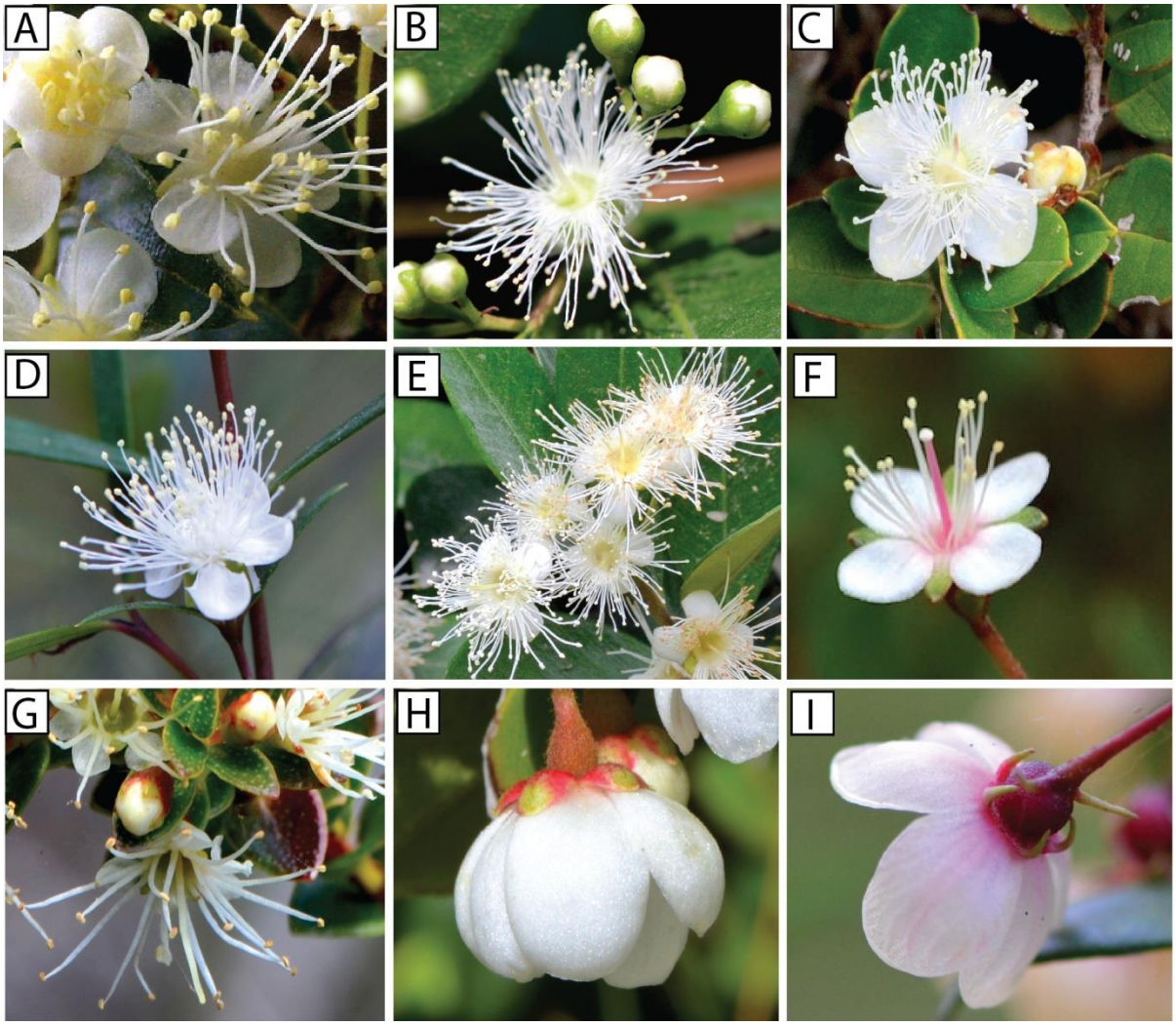


Figure 5.1. Gross morphology of flowers of Chilean Myrtaceae. A, *Amomyrtus luma*. B, *Blepharocalyx cruckshanksii*. C, *Luma apiculata*. D, *Myrceugenia lanceolata*. E, *Myrceugenia planipes*. F, *Myrteola nummularia*. G, *Metrosideros stipularis*. H, *Ugni candollei*. I, *Ugni molinae*. Tetramerous species: B, C, D, E, F. Pentamerous species: A, G, H, I.

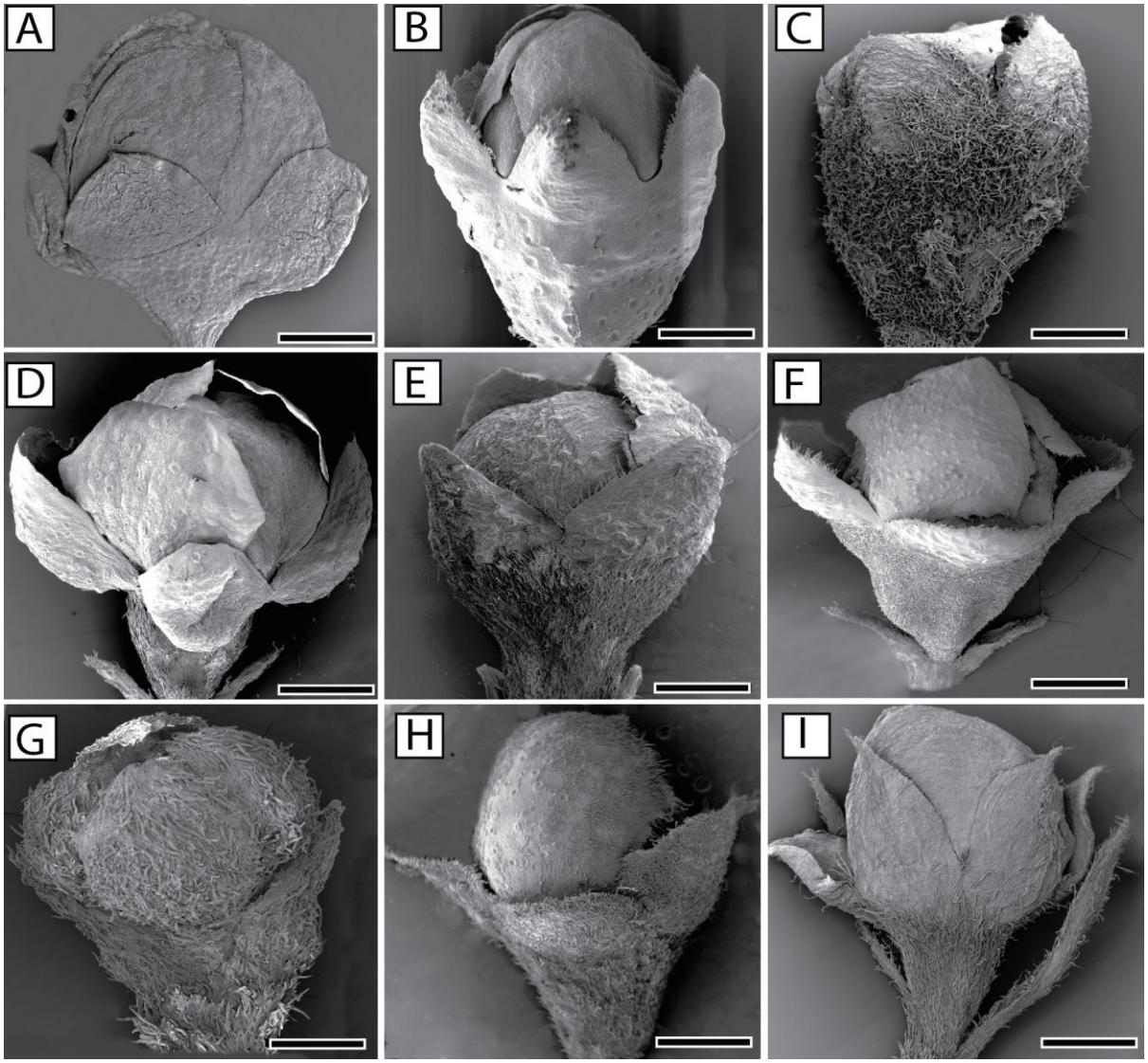


Figure 5.2. Scanning electron micrographs (SEM) of flower buds in Chilean Myrtaceae. A, *Luma apiculata* showing sepals, petals and hypanthium essentially glabrous or having a sparse layer of hairs. B, *Metrosideros stipularis* showing glandular and glabrescent sepals petals and hypanthium. C, *Myrceugenia colchaguensis* showing petals, sepals and hypanthium densely covered with hairs. D, *Myrceugenia parvifolia* showing sepals, petals, hypanthium and bracteoles with sparse hairs E, *Myrceugenia pinifolia* showing a sparse layer or hairs. F, *Myrceugenia planipes* showing sepals, petals, hypanthium and bracteoles with a sparse layer of hairs. G, *Myrceugenia rufa* showing petals, sepals and hypanthium densely covered with hairs. H, *Myrcianthes coquimbensis* showing petals, sepals and hypanthium densely covered with hairs. I, *Ugni candollei* showing petals, sepals, hypanthium and obvious bracteoles densely covered with hairs. Scale bars = 5mm.

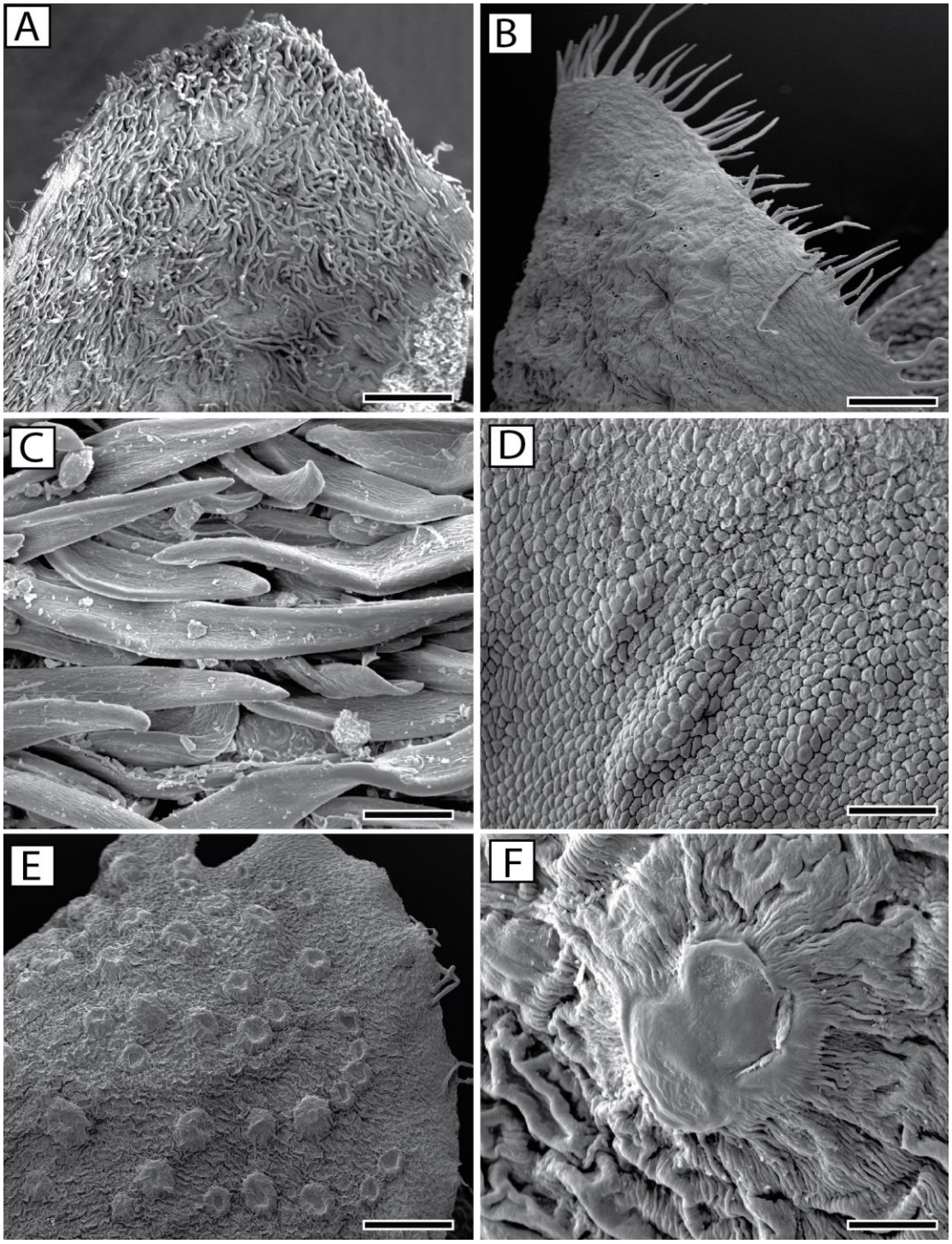


Figure 5.3. Scanning electron micrographs (SEM) of flower elements in Chilean Myrtaceae. A, abaxial surface of sepal with a dense layer of simple hairs in *Blepharocalyx cruckshanksii*. B, long simple hairs on the margin of the sepals in *Luma chequen*. C, dense layer of dibrachiate hairs on sepals of *Myrceugenia rufa*. D, epidermal cells of sepals with slightly undulating-straight anticlinal walls in *Myrcianthes coquimbensis*. E, sepal showing abundant secretory cavities and sparse hairs on margin in *Myrceugenia obtusa*. F, detail of secretory cavity on the sepals in *Luma chequen*. Scale bars = 100 µm (A,B,E), 10 µm (C,D,F).

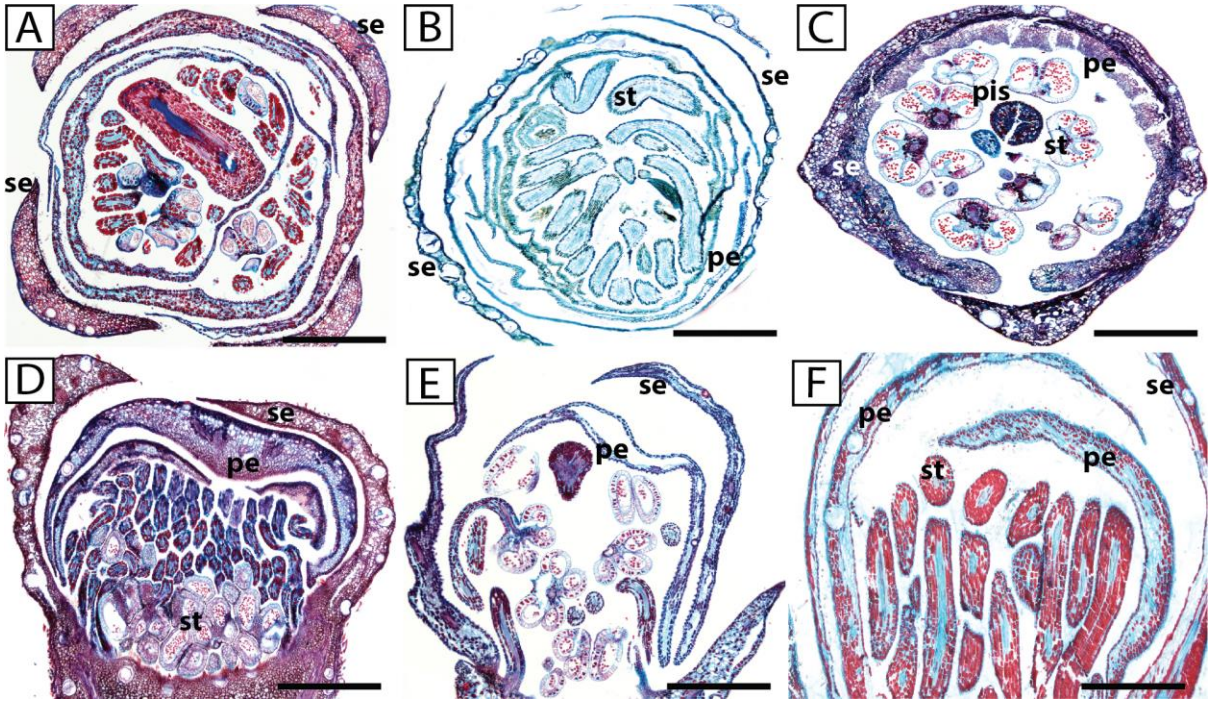


Figure 5.4. Light micrographs (LM) of transverse (A-C) and longitudinal (D-E) sections of the anatomical organization of flower buds in species of Chilean Myrtaceae. A, *Blepharocalyx cruckshanksii* showing thick sepals with abundant secretory cavities and thin petals with cellular content. B, *Luma chequen* showing thin sepals and petals with large secretory cavities. C, *Metrosideros stipularis* showing thick sepals with large secretory cavities. D, *Myrceugenia exsucca* showing thick sepals and petals. Sepals have abundant secretory cavities. E, *Metrosideros stipularis* showing thin sepals and petals. F, *Ugni molinae* showing thick sepals and petals with abundant secretory cavities and abundant cellular content. Scale bars = 250 µm. se: sepals, pe: petals, st: stamens, pis: pistil. Stains used: Ruthenium red-TBO (A,C,D,E), TBO (B), safranin-alcian blue (F).

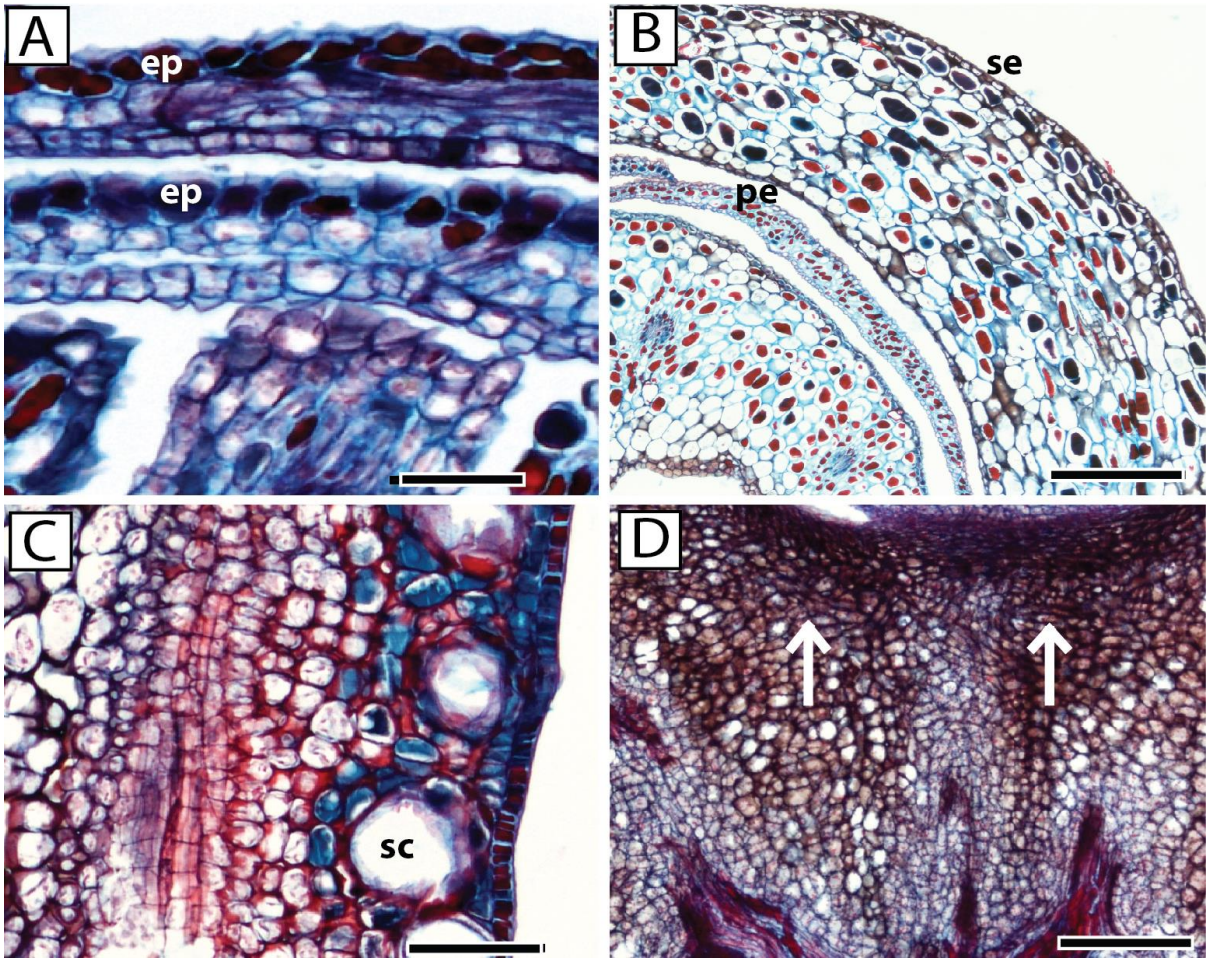


Figure 5.5. Light micrographs (LM) of transverse (A) and longitudinal (B-D) sections showing anatomical elements in the perianth and hypanthium of Chilean Myrtaceae. A, abundant tannins in the epidermis and some mesophyll cells from the petals in *Myrceugenia exsucca*. B, Numerous cell layers in the sepals and few layers in the petals of *Ugni candollei*. C, secretory cavities in contact with the epidermis of the hypanthium in *Amomyrtus luma*. D, conspicuous nectariferous region in the hypanthium of *Myrcianthes coquimbensis* (white arrows). ep: epidermis of petals, pe: petals, se: sepals, sc: secretory cavities. Stains used: Ruthenium red-TBO (A,B), safranin-alcian blue (C,D). Scale bar = 25 µm.

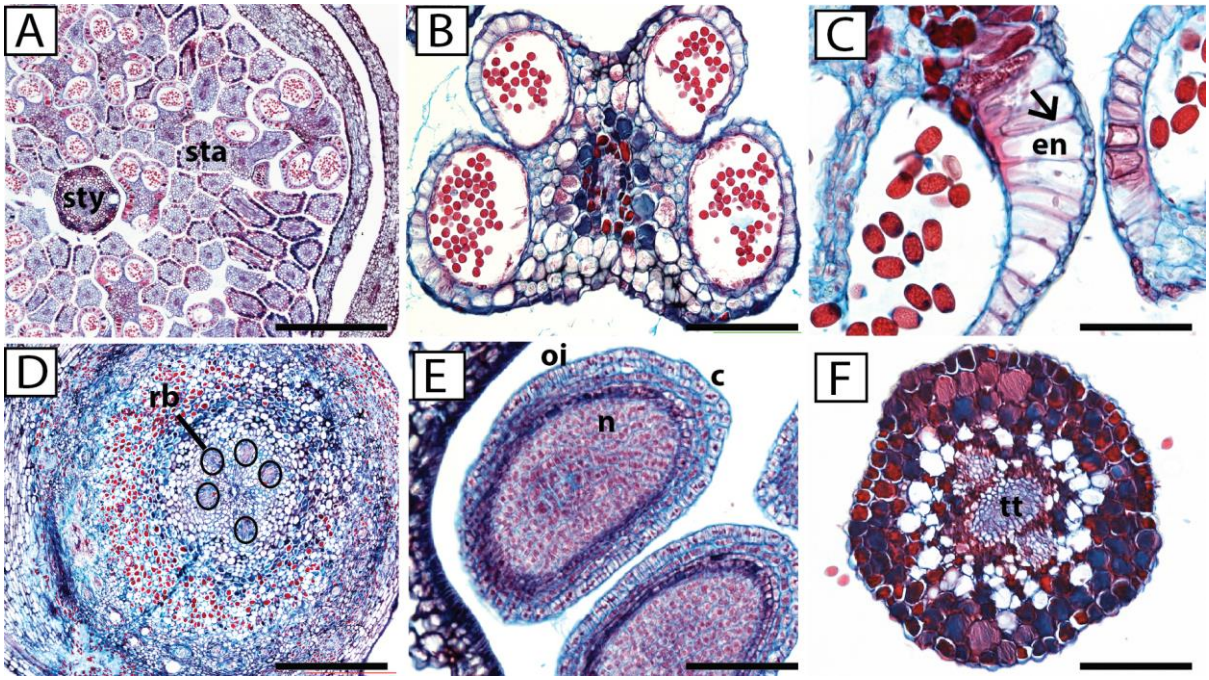
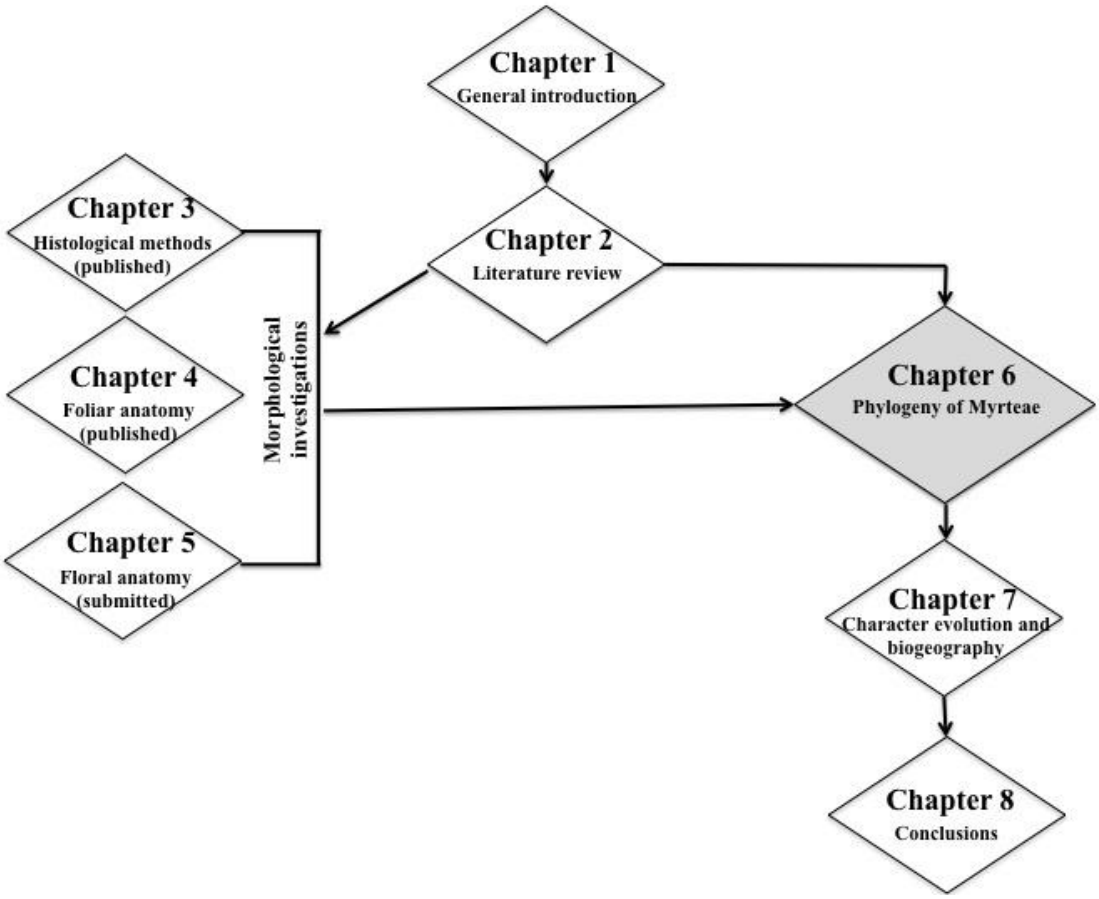


Figure 5.6. Light micrographs (LM) of anatomical elements in androecium (A-C) and gynoecium (D-F) of Chilean Myrtaceae. A, numerous stamens in *Blepharocalyx cruckshanksii*. B, anther showing connective tissue with abundant cellular content and thecae containing pollen grains in *Luma apiculata*. C, detail of endothecium showing strips of secondary thickenings on the outer periclinal walls in *Luma apiculata*. D-F, gynoecium: D, ovary showing five receptacular bundles in *Myrceugenia planipes*. E, detail of ovules showing outer integuments in *Myrteola nummularia*. F, abundant tannins in different cell layers of the style in *Luma apiculata*. c: chalaza, en: endothecium (black arrow), n: nucellus, oi: outer integument, rb: receptacular bundles (polygons), sta: stamens, tt: transmitting tissue. Stains used: Ruthenium red-TBO (A,D,E), alcian blue-safranin (B,C,F). Scale bars = 100 µm (A), 25 µm (B,E), 50 µm (D), 10 µm (C,F).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CHAPTER 6: Phylogenetic position of the Chilean Myrteae (Myrtaceae) using morphological and molecular data

Abstract

The tribe Myrteae (Myrtaceae) is represented in Chile by 25 species in nine genera, many of which have restricted geographic distribution and an endangered conservation status. A phylogenetic investigation of the tribe Myrteae (Myrtaceae), based on sequences of two nuclear (ETS, ITS), two plastid DNA regions (*matK*, *psbA-trnH*) and 79 morphological characters is presented. Bayesian, maximum parsimony and maximum likelihood analyses were conducted. Taxonomic selection included 111 species representing 32 genera of Myrteae, 10 outgroup species and all the 25 Chilean species. Some species such as *Myrcianthes coquimbensis*, *Ugni candollei*, *Ugni myricoides* and representatives of the Australian *Austromyrtus*, *Lenwebbia*, *Lithomyrtus* are included for the first time in a phylogenetic study. This is the first phylogenetic study using morphological characters at the tribal level for reconstruction of relationships. Phylogenetic results agree with previous studies and confirm the monophyly of the tribe Myrteae in all analyses and the position of the Mediterranean *Myrtus* as sister to all other Myrteae in most analyses. The Chilean Myrteae are distributed among six main clades. *Amomyrtus*, *Luma* and *Myrceugenia* are monophyletic, while *Ugni* and *Blepharocalyx* are not monophyletic in most of the molecular analyses. The four species of *Ugni* occur in two clades (*Ugni molinae* + *Ugni myricoides* and *Ugni selkirkii* + *Ugni candollei*) nested in a clade with *Myrteola*, *Neomyrtus*, *Lophomyrtus* and *Lenwebbia* in most analyses. The Chilean *Myrcianthes coquimbensis* is sister to all other species of *Myrcianthes* with strong statistical support. The Chilean Myrteae are sister to major South American and New Zealand-Australian clades. Morphological characters provided resolution and improved statistical support in some clades when combined with molecular data. Future phylogenetic analyses with a more comprehensive sampling of Myrteae, particularly *Myrcianthes* and *Blepharocalyx*, are recommended.

6.1 Introduction

The tribe Myrteae (Myrtaceae) is one of the most abundant taxonomic groups in Australasian and South American rainforests (Lucas et al., 2007; Biffin et al., 2010). Australasian genera

such as *Austromyrtus*, *Decaspermum*, *Gossia*, *Lenwebbia*, *Rhodomyrtus* and *Rhodamnia* mainly occur in the rainforests of eastern Australia and Southeast Asia (Appendix 3). Predominant South American rainforest species include *Acca*, *Calyptranthes*, *Eugenia*, *Gomidesia*, *Myrcia*, *Pimenta*, *Psidium* and *Siphoneugena* (Lucas et al., 2007; Wilson, 2011). All the South American species of the family Myrtaceae belong to the tribe Myrteae, except for the Chilean-Argentinean *Metrosideros stipularis* (syn. *Tepualia stipularis*), which is in Metrosidereae (Pillon et al., 2015).

In Chile, the tribe Myrteae is represented by 25 species (plus one variety) in nine genera (Landrum, 1988a). Chilean Myrteae are distributed from north-central Chile to the southern tip of Chile-Argentina and in the Juan Fernandez Islands. They inhabit humid temperate forests and other habitats such as coastal xeromorphic formations and shrublands (Landrum, 1988a; Landrum and Grifo, 1988; Hildebrand-Vogel, 2002). Four genera of Chilean Myrteae (*Amomyrtus*, *Legrandia*, *Luma* and *Nothomyrcia*) are endemic to the humid temperate forests of Chile and Argentina. *Amomyrtus* and *Luma* have two species each, while *Legrandia* and *Nothomyrcia* are monospecific genera (Landrum, 1988a). *Legrandia concinna* has a very restricted geographic distribution in southern Chile and has an endangered conservation status (Benoit, 1989). *Nothomyrcia fernandeziana* is endemic to the Juan Fernandez Islands (Murillo and Ruiz, 2011). The remaining five genera have a broader geographic distribution range and occur outside of Chilean-Argentinean forests. The genus *Ugni* has four species, two of which occur in the forests of mainland Chile, one is endemic to Juan Fernandez Islands and one occurs in Mexico and Central America (Wilson, 2011). *Blepharocalyx* has three species, of which one is endemic to Chile and the other two occur in the Caribbean, Brazil, Paraguay, Uruguay and Argentina. *Myrcianthes* includes ca. 30 species, with one species endemic to Chile and the remaining distributed in the Andes from Mexico to Peru and eastern South America (Wilson, 2011). *Myrteola* includes three species, of which one occurs in Chile, Argentina, Peru, Colombia and Venezuela, while the other two species occur in Colombia and Venezuela (Landrum, 1986, 1988b). The genus *Myrceugenia* has ca. 40 species, of which nine species occur exclusively in continental Chile, three species (and one variety) in central-southern Chile and Argentina, one species is endemic to the Juan Fernandez Islands and ca. 17 species occur in southeast Brazil (Landrum, 1981a) (Appendix 3). The number of genera in Chilean Myrteae is particularly high compared to the number of species, which has been postulated as a product of early isolation of the Chilean flora and the existence of natural barriers (Villagrán and Hinojosa, 1997; Moreira-Muñoz, 2011).

However, some authors have indicated that linking number of species/genera to processes that drive biodiversity should be cautious (Gravel et al., 2011; Foote, 2012). Most of the species of Chilean Myrteae are endangered, vulnerable or have other status of conservation concern (Benoit, 1989).

The systematic position of Chilean Myrteae has been determined as sister group to eastern South American or Australasian genera (Lucas et al., 2007; Murillo et al., 2013). In Lucas et al. (2007), *Myrceugenia*, *Blepharocalyx* and *Luma* formed a monophyletic group ("*Myrceugenia* group") sister to a large South American clade including *Myrcia*, *Calyptranthes* and *Gomidesia* ("Myrcia group"). The monophyly of the "*Myrceugenia* group" has been questioned, since the three included genera have been suggested as part of different lineages (Murillo et al., 2013, 2016). The phylogenetic position of *Myrceugenia rufa* has been determined as sister to all the other species of the genus, *Ugni selkirkii* (Alejandro Selkirk Island, Juan Fernandez Archipelago) sister to *Myrceugenia* and the bitypic *Luma* as sister to all species of Myrteae, except for *Myrtus communis* (Murillo et al., 2013). The systematic position of the two Chilean varieties of *Myrceugenia ovata* (*var. ovata* and *var. nanophylla*) needs clarification, since the two varieties are sister in some studies (Murillo et al., 2016) but not in others (Landrum, 1981a; Murillo et al., 2013). *Ugni molinae* was sister to the New Zealand *Lophomyrtus* and *Neomyrtus* (Lucas et al., 2007; Murillo et al., 2013). *Myrteola nummularia* is either sister to the Australasian species (Murillo et al., 2013) or sister to a clade containing the New Zealand species and *Ugni molinae* (Lucas et al., 2007). The phylogenetic position of *Amomyrtus+Legrandia* has been determined as sister to all the other members of the South and Central American "*Pimenta* group" (Lucas et al., 2007; Murillo et al., 2013). The monophyly and systematic position of the Chilean genera of Myrteae is unclear, as species such as *Ugni candollei* and *Myrcianthes coquimbensis* have not been included in any prior phylogenetic analysis. Phylogenetic analyses of Myrteae have generally shown weak support and poor resolution of many clades. The sister positions of Chilean Myrteae to other major clades need more clarification and statistical support in order to discuss biogeographic implications of these relationships.

The utility of morphological data (e.g., gross morphology, micromorphology, anatomy) in phylogenetic analyses has been indicated in several studies, particularly combined with molecular data (Baum, 1989; Lens et al., 2007; Doyle and Le Thomas, 2012). The inclusion of morphological characters in phylogenetic reconstruction might contribute to increase resolution and statistical support of trees when characters are informative (Scharaschkin and

Doyle, 2006; Prevosti and Chemisquy, 2010). Some plant families where morphological characters have provided valuable information for phylogenetic reconstruction include Annonaceae (Scharaschkin and Doyle, 2005), Orchidaceae (Ponsie et al., 2007), Poaceae (Ruiz-Sanchez et al., 2008), Bonnetiaceae and Hypericaceae (Ruhfel et al., 2013). Histochemical characters have been regarded as potentially informative for phylogenetic analyses, especially combined with anatomical and gross morphological characters (Schmid, 1980; Judd et al., 2008; Wink, 2011). The identification and interpretation of histochemical characters strongly relies on the protocol used and its applicability to a certain family/group of plants (Johansen, 1940; Ciccarelli et al., 2008). The utility of morphology for phylogenetic reconstruction is uncertain in the tribe Myrteae, since its inclusion has yet to be comprehensively conducted at tribal level. Anatomical information is scarce in Myrteae and especially in Chilean species, which prevents the construction of a robust morphological data set for phylogenetic analyses. A number of morphological characters have been considered important for classifying some genera of Chilean Myrteae, namely type of inflorescence, type of hairs, number of sepals and petals, caducity of bracteoles and type of embryo (Landrum, 1988a). Morphological characters have been also indicated as potentially informative to reconstruct relationships in the tribe, such as type of perforation plates, helical wall thickenings on vessels, placentation and some floral anatomical characters (Schmid, 1972, 1980; Wilson et al., 2001; Lucas et al., 2007). It has been indicated that scoring of homologous morphological characters in Myrteae/Myrtaceae might be a difficult task and that should be conducted based on extensive research and observation (Lucas et al., 2007; Vasconcelos et al., 2015). The comprehensive evaluation of morphological characters as a potential source of phylogenetic information in Myrteae has not been undertaken.

Due to conflicting results between earlier phylogenetic studies, the low support and poor resolution observed in a number of clades, the unknown position of *Myrcianthes coquimbensis* and the unclear relationships of *Ugni* and other Chilean genera, a phylogenetic analysis of Myrteae with a comprehensive sampling of Chilean genera is presented. In this study, phylogenetic analyses were based on DNA sequences and a comprehensive data set of morphological characters.

6.2 Material and methods

6.2.1 Taxonomic sampling and sample acquisition

A total of 111 species were included in the phylogenetic analyses. Taxonomic sampling was designed to represent all the major clades identified in previous phylogenetic studies in Myrteae (Lucas et al., 2007; Murillo et al., 2013, 2016), as well as intertribal studies for the outgroup (Biffin et al., 2010; Thornhill et al., 2015). All 26 species of Chilean Myrtaceae were analysed, including 25 species of Myrteae in the ingroup and one species in the outgroup as representative of *Metrosideros* (Metrosidereae). Outgroup species were representatives from seven tribes of the subfamily Myrtoideae (Syzygieae, Metrosidereae, Eucalypteae, Leptospermeae, Backhousieae, Tristanieae, Kanieae), with phylogenetic relationships well established in previous studies (Wilson et al., 2005; Lucas et al., 2007; Biffin et al., 2010). Taxonomic sampling of the ingroup represents 32 of the 53 Myrteae genera recognised by Wilson (2011). Two Chilean species (*Myrcianthes coquimbensis* and *Ugni candollei*) have been included here for the first time in a phylogenetic analysis. The South American species *Ugni myricoides* was also included to test the monophyly of the genus *Ugni* including all the four species. Additional Australasian species (*Austromyrtus* spp., *Lenwebbia* spp., *Lithomyrtus* spp.) were added to this sampling for a better representation of the Australasian clade found in previous studies (Lucas et al., 2007; Murillo et al., 2013). A total of 83 accessions were used for morphological descriptions and production of DNA sequences. Samples for DNA extraction and/or morphological descriptions were either collected fresh or obtained from herbarium specimens (BRI, NSW, CONC). Vouchers of Chilean Myrteae collected in this study are currently deposited in the Faculty of Forest Sciences Herbarium, University of Chile (EIF), with duplicates housed in the Queensland Herbarium, Brisbane, Australia (BRI).

6.2.2 Morphological data

Freshly collected material for morphological descriptions was fixed in FAA and then dehydrated using a graded ethanol series. Herbarium material was soaked in boiling water for 10 min to recover the leaf shape before being fixed in FAA and dehydrated (Haron and Moore, 1996). Samples for SEM observations were treated using the guidelines of Anderson (1951) using critical point drying in an Autosamdri-815 automatic critical point drier (Tousimis, Rockville, USA). Samples were mounted on stubs with self-adhesive double-sided carbon discs and sputter-coated with gold palladium for 175 sec using a Leica EM

SCD005 Gold Coater (Leica Microsystems, Macquarie Park, NSW, Australia). Examination and documentation of images was carried out using a FEI Quanta 200 SEM/ESEM (FEI, Hillsboro, Oregon, USA) operated at 10kV.

Samples for anatomical observations were embedded in paraffin wax (Johansen, 1940; Ruzin, 1999). Transverse sections of leaves were cut using a Leica RM2245 rotary microtome (Leica Microsystems, Macquarie Park, NSW, Australia) at 5µm. Staining of sections was performed using the stains Ruthenium red (0.05% aqueous solution), Toluidine blue (TBO) (0.1% aqueous solution), Safranin O (1% alcoholic solution) and Alcian blue, alone or combined according to standard staining protocols (Ruzin, 1999; Retamales and Scharaschkin, 2014). Sudan IV, Chlorazol black E and Phloroglucinol (20% HCl) were used to detect lipophilic substances and lignin. Chemical nature of leaf intracellular crystals was tested by adding 1µl of acetic acid and 1µl of hydrochloric acid to sections (Maclean and Ivimey-Cook, 1952). Sections were mounted using DPX (Sigma-Aldrich Co., St. Louis, Missouri, USA).

Leaf clearings were prepared by immersing 1-2 cm^2 pieces of leaf material in 10% KOH at room temperature for 48 h followed by 7% NaClO for 2 h or until leaves turned transparent (Gardner, 1975). Cleared leaves were washed five times with distilled water, stained with 1% safranin O and mounted with Lactoglycerol (lactic acid-glycerol 1:1). Slides were observed using a Nikon eclipse 50i compound microscope and images captured using the Nikon NIS-Elements imaging software (Nikon Instruments Inc., Amsterdam, Netherlands).

6.2.3 Molecular data

Selection of DNA loci

Two nuclear and two chloroplast loci were targeted for amplification and sequencing for the phylogenetic analyses. Sequences of the nuclear ribosomal internal transcribed spacer (ITS), external transcribed spacer (ETS), the maturase K (*matK*) gene and the plastid *psb*A-*trn*H intergenic spacer were selected based on previous analyses on suprageneric and infrageneric relationships in Myrtaceae and Myrteae (Lucas et al., 2005, 2007; Biffin et al., 2010; Murillo et al., 2013). Target DNA regions were selected on the basis of their phylogenetic information to reconstruct relationships in the tribe Myrteae and availability of data. The use of nuclear loci (mainly ITS and ETS) has been proven informative in many studies of Myrtaceae (Lucas et al., 2005, 2007; Biffin et al., 2007, 2010; Soh and Parnell, 2011; Snow et al., 2012; Murillo et al., 2013). The chloroplast non-coding region *psb*A-*trn*H has not been

used by many authors but has proven to resolve infrageneric relationships in Myrteae (Lucas et al., 2007, 2011; Mazine et al., 2014). Non-coding regions have been regarded as more useful at species level, since these target regions are known for having higher substitution rates (Clegg et al., 1994). Previous authors (Lucas et al., 2007; Mazine et al., 2014) have indicated that ETS, ITS, *matK* and *psbA-trnH* regions are suitable for combined analyses in Myrteae because of lack of evidence of hybridization and horizontal transfer.

DNA extraction, amplification, sequencing and data set assembly

Genomic DNA was extracted from approx. 0.5-0.8 g of fresh, silica gel preserved or herbarium-specimen leaves. DNA extraction was conducted using either a modified version of the CTAB (hex-adecyltrimethyl ammonium bromide) extraction protocol (Doyle and Doyle, 1987) or using the DNA Isolate II Plant DNA kit following manufacturer's directions (Bioline Aus. Pty Ltd, Alexandria, New South Wales, Australia). Modifications of the CTAB method included RNAse treatment (Healey et al., 2014) and 100% ethanol precipitation for 5-8 weeks. Activated charcoal suspension was also used for older and deteriorated herbarium specimens (Krizman et al., 2006). Primers used for amplification and sequencing and PCR conditions were mainly based on Lucas et al. (2007) with some modifications (Table 6.1). PCR reactions consisted of 28 µl reactions, containing 16.9 µl of double distilled water, 5 µl of reaction buffer including dNTPs (200mM Tris-HCl, 500mM NH_4, 200mM dNTP), 2 µl of each forward and reverse primers (10 uM), 2 µl of $MgCl_2$, (100 µM) 0.1 µl of *Taq* DNA polymerase (5U/ µl) (Bioline Aus. Pty Ltd, Alexandria, New South Wales, Australia) and 1 µl (approx. 20 ng/ µl) of genomic DNA. Unsuccessful PCR amplifications were repeated using 2 µl (>20 ng/ µl) of DNA in the PCR reactions instead of 1 µl. Myrtaceae are known for being rich in secondary compounds and metabolites such as polysaccharides and tannins (Retamales and Scharaschkin, 2014), which often inhibit PCR amplification. A number of methods were explored in order to overcome inhibited PCR amplifications, including addition of TBT-PAR reagent (trehalose, bovine serum albumin and polysorbate-20) (Samarakoon et al., 2013) and dimethyl sulfoxide (DMSO) (Murillo et al., 2013) to the PCR reactions.

Amplification of target regions was conducted in an Eppendorf 6325 gradient thermal cycler (Eppendorf South Pacific Pty. Ltd., North Ryde, New South Wales, Australia). PCR products were sent to Macrogen (Seoul, Korea) for purification and bidirectional sequencing with the same primers used for PCR amplification. Sequencing was carried out under BigDye™

terminator cycler conditions at Macrogen, while products were purified with ethanol precipitation and run under an automatic sequencer ABI3730XL. Twenty-nine sequences were newly produced for this study for three of the loci (ETS, ITS and *psbA-trnH* intergenic spacer) (Appendix 6.2) and submitted to GenBank (Table 6.2). Most DNA sequences were sourced from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) as part of previous phylogenetic investigations in Myrteae and Myrtaceae (Lucas et al., 2007; Biffin et al., 2010; Lucas et al. 2011; Murillo et al., 2012, 2013, Mazine et al., 2014). An expanded molecular dataset containing 179 species and representing all the available GenBank sequences of the large genera *Myrcia* and *Eugenia* was also analysed. The purpose of this expanded analysis was to test the effect of taxon sampling in the systematic position of *Myrcianthes coquimbensis*, since the genus *Myrcianthes* has been proposed as closely related to *Eugenia*. Some *matK* sequences from GenBank were not included in this investigation as they were deemed low quality (e.g., *Myrceugenia chrysocarpa* - JN660995, *Myrceugenia euosma* - JN660998, *Myrceugenia lanceolata*-JN661007).

ABI sequence trace files were assembled in Geneious R9 (Biomatters Ltd.) and aligned using the package MUSCLE under default parameters (Re-align sequences, max. number of iterations=8, anchor optimization). Additional adjustments were made by eye in Geneious R9 based on the guidelines of Kelchner (2000) and Morgan and Kelchner (2010). Ambiguously aligned blocks were excluded from the analyses. Gaps were considered missing data. Parsimony-informative, insertions and deletions (indels) were scored as binary characters (Table 6.3) following the coding method of Simmons and Ochoterena (2000).

6.2.4 Morphological data set for phylogenetic reconstruction

Morphological characters used in earlier studies formed the basis for the starting point of creating a morphological data set (Schmid (1972), Johnson and Briggs (1984), Landrum (1981, 1988a, 1988b), Wilson et al. (2001), Lucas et al. (2007), Cardoso et al. (2009), Soh and Parnell (2011), Pimentel et al. (2014) and Vasconcelos et al. (2015)). Selection of characters and character states was re-evaluated and modified, if needed, given the current taxon sampling. Additional characters were added based on personal observations and publications (Retamales et al., 2014a, 2014b; Retamales and Scharaschkin, 2015). A few potentially informative characters for Myrteae were examined for the first time (e.g., conspicuous nectariferous region, secretory cavities in petals, multiple epidermis).

Those characters that showed variation between outgroup species were included in the data matrix regardless of their variation in Myrteae. Delimitation of character states was conducted following the guidelines of Wiens (2000) and Stevens (1991). Characters examined, but later excluded due to extreme intraspecific variation or difficulty in assessing homology, were average stomatal density, average density of secretory cavities, cuticle thickness and density of hairs. The use of quantitative characters for phylogenetic analyses has been criticized by some authors (Stevens, 1991) mainly due to ambiguity. No quantitative characters were scored in this investigation. Polymorphic characters were managed according to the guidelines provided by Wiens (1995, 1999), where the most common state was selected. Detailed justification, documentation, scoring, modified characters and references for morphological characters are presented in Chapter 7. The resulting morphological dataset consisted of 79 parsimony-informative qualitative characters (Appendix 2), of which 49 are binary characters and 30 are unordered multistate; 22.3% are scored as missing data (Table 6.4). This ratio of missing morphological entries is within acceptable limits for phylogenetic analyses (Prevosti and Chemisquy, 2010). Characters and character states were compiled in Mesquite v3.10 (Maddison and Maddison, 2016). Character states were annotated as entire numbers (e.g., 0, 1). Unknown (?) means that the character state is not available for that species or is not possible to be unambiguously interpreted (Table 6.4).

6.2.5 Phylogenetic reconstruction

Molecular data sets were analysed individually and then as a combined data set (molecular analyses). The morphological data set was analysed alone and then in combination with the molecular dataset (total evidence analyses). When two or more datasets were combined, any species present in one and missing in the other was scored as a question mark line as missing data (Sauquet et al., 2003). Relationships based on morphology alone and on faster-evolving DNA sequences appear to be sensitive to the present taxon sampling. Due to this reason, the outgroup relationships were constrained to the topologies found by Biffin et al., (2010) and Thornhill et al. (2015), but the monophyly of the tribe Myrteae was not enforced. Constraint trees were constructed using the tree building option in Mesquite V3.10 (Maddison and Maddison, 2016)

Bayesian analyses

Bayesian inference (BI) was implemented in MrBayes v3.2.3 (Ronquist et al., 2012). The best-fitting models of DNA substitution were determined by jModelTest 2 (Darriba et al.,

2012) for all molecular partitions using the Akaike Information Criterion (AIC). The selected models were the TVM+G, GTR+I+G, TVM+G and TVN+G for ETS, ITS, *matK* and *psbA-trnH* respectively. These different models were applied independently to each molecular partition. Six substitution types (nst=6) and a gamma distribution (rates=gamma) were required for all models and specified in the command blocks. Rate heterogeneity, substitution rates and frequency of bases across were unlinked across the partitions. Substitution models largely agree with those used in Lucas et al. (2007). Analysis of morphological data was conducted using the likelihood model for discrete morphological characters and indicating that only characters with variation among species were included (coding=variable) (Ronquist et al. 2012). The models used in BI and ML for indel data were the MrBayes v3.2.3 default for standard data and the standard model respectively. A simple symmetric model with equal frequency of states and a variable relative rate (ratepr=variable) for branch lengths, were selected for the morphological data set (Ronquist et al., 2012). All Bayesian analyses consisted of a Markov Chain Monte Carlo process (Huelsenbeck and Ronsquist, 2001) conducted with two concurrent runs of four incrementally heated chains for 50 million generations, sampled every 1000 generations. The temperature coefficient was set at 0.5 to promote mixing between the four chains. Convergence of the Markov Chain Monte Carlo runs and burn-in values were verified using the software Tracer v1.6 ensuring that effective sample sizes of each parameter was greater than 200 (Rambaut et al., 2014). The first 25% of the trees from the two runs were excluded as burn-in before building of consensus trees. Bayesian posterior probabilities (PP) were used as statistical support.

Maximum likelihood analyses

Maximum likelihood (ML) analyses were conducted using RaxML v8.1.11 (Stamatakis, 2014) for molecular, morphological and total evidence using the same models of evolution as in Bayesian analyses. Statistical robustness of clades was estimated using non-parametric bootstrap support (BS) (Felsenstein, 1985) of 1000 replicates.

Maximum parsimony analyses

Maximum parsimony (MP) analyses were conducted under the Fitch (1971) criterion (unordered and equally weighted characters) implemented in PAUP* version 4.0b10 (Swofford, 2002). Parsimony analyses were carried out for the morphological, molecular and total evidence data sets. Heuristic searches were conducted with 1000 replicates using random taxon addition and TBR (tree bisection and reconnection) branch swapping in order

to recover the most-parsimonious trees (MPTs). A maximum of 50,000 trees per analysis was established. Bootstrap analysis (Felsenstein, 1985) was used to estimate the relative statistical support of clades recovered by the parsimony analyses. A total of 1000 bootstrap replicates were run using heuristic search parameters and TBR branch swapping, with a limit of 10,000 trees.

Evaluation of statistical support

Bootstrap values from 50 to 70 were considered weak support, 71 to 85 were considered moderate support and 86 to 100 were considered as strong support (Hillis and Bull, 1993). Values under 50 BS were not shown. As an additional measurement of support (not statistical) the recovery percentage for clades in consensus trees (i.e., 100% -strict consensus, 50% - majority rule) is provided. Most of the phylogenetic trees illustrated in this investigation are majority rule trees. Majority rule trees and strict consensus trees from MP and BI were generated and visualized in Mesquite v3.10. Maximum likelihood best-scored trees were viewed using Figtree v.1.4.2 (Rambaut, 2012). Bayesian posterior probabilities (PP) and bootstrap support (BS) were mapped onto generated trees. Trees were visualized and edited for presentation using Figtree and Adobe illustrator CC 2014.

6.3 Results

Statistics from the MP phylogenetic analyses for each locus, including scored indels, proportion of variable characters, number of trees generated and parsimony-informative characters are summarized in Table 6.5. Since statistically supported incongruences (BS>70) were generally not found among trees from individual loci, data sets were combined (Table 6.5). Constraint outgroups did not impact the phylogenetic relationships and statistics of the analyses. Indel coding slightly improved the resolution of some clades but had not a significant effect in the overall topology and statistics. Only results from the original taxon sampling (111 species) with coded indels will be discussed henceforth. Results from the expanded analysis including all available sequences of *Eugenia, Myrceugenia* and *Myrcia* (179 species) will be mentioned when relevant.

6.3.1 Molecular data sets

Phylogenetic analyses of individual molecular data sets (ETS, ITS, *psbA-trnH*, *matK* individually) yielded trees with poor resolution and weak statistical support. Phylogenetic trees obtained from the nuclear data set (combination of the two nuclear loci; ETS, ITS) and

chloroplast data set (combination of the two chloroplast loci; *psbA-trnH, matK*) had improved statistical support and resolution. Trees obtained from nuclear and chloroplast data sets were generally more resolved in parsimony and maximum likelihood analyses (Fig. 6.1). Topologies based on the nuclear or chloroplast data set were generally similar across the three methods (BI, MP, ML) with no well-supported incongruences in the phylogenetic position of genera (Fig. 6.1). In general, consistency indices (CI) are moderately low in most reconstructions (Table 6.5). The combined molecular data set (nuclear and chloroplast data sets combined) provided more resolved phylogenetic trees (Fig. 6.3) and with a number of clades moderately to strongly supported (Table 6.6).

6.3.2 Morphological data set

Phylogenetic trees obtained from BI, MP and ML using only morphological data were very similar (Fig. 6.1) and had low statistical support (Table 6.6), but were fairly resolved. In all the trees, several monophyletic groups were obtained, including a number of genera with Chilean representatives (e.g., *Myrceugenia, Luma, Amomyrtus*) (Table 6.6). Major clades recovered using only morphological data were similar to those recovered using molecular data, with some clear differences in the phylogenetic position of some genera (e.g., *Plinia*, *Nothomyrcia*, *Myrtus*). Consequently, on the basis of the good resolution and phylogenetic position of major clades across the three methods, the morphological data set was combined with the molecular data set to infer phylogenetic relationships.

6.3.3 Combined molecular and morphological data sets (total evidence)

The phylogenetic structure between the total evidence analyses using BI and ML are very similar, while trees generated using MP are less resolved. The total evidence analyses (Figs 6.5, 6.6) provided the best resolution and higher statistical support in many clades (Table 6.6) using the three phylogenetic methods, but particularly Bayesian Inference (BI). The fewest most parsimonious trees were produced from the total evidence analysis (272), compared to all other analyses (>50,000 trees).

6.4 Discussion

The following section will discuss the phylogenetic relationships within Myrteae in light of the results obtained in this study and previous investigations. The discussion will be based on the phylogenetic relationships obtained from the combined molecular (four loci) and the total evidence analyses (four loci plus morphology), since these analyses provided the best

resolution and strongest statistical support using the three methods (MP, BI, ML). The discussion will be organized by major relationships at tribal level and then will focus on the phylogenetic position of Chilean genera.

6.4.1 Overall phylogenetic relationships within the tribe Myrteae

Results in this investigation reinforce the findings from previous investigations where individual loci generally do not yield resolved and strongly supported trees, mainly because of a lack of enough characters to overcome homoplasy (Sauquet et al., 2003; Jewell et al., 2012; Murillo et al., 2012). Lucas et al. (2007) interpreted this as a possible lack of phylogenetic signal in Myrtaceae, especially for ITS resolving internal branches. According to Sauquet et al. (2003), the combination of data sets enables the cancelation of conflicting sources of noise from individual loci, since the signal from a common evolutionary history is additive. It seems that a large number of characters have to be analysed simultaneously in order to yield strongly/moderately supported relationships in Myrteae, which has been observed in other plant groups (Soltis et al., 1998, Hoot et al., 1999; Sauquet et al., 2003; Scharaschkin and Doyle, 2006). However, the study of informative individual loci might be important to look for congruent signals across markers in order to further explore phylogenetic relationships in Myrtaceae.

The phylogenetic structure obtained in this study largely agrees with previous tribal phylogenetic studies on Myrteae (Lucas et al., 2007; Murillo et al., 2013, 2016). The tribe Myrteae was monophyletic in all analyses (PP 1.0, BS 98-100) (Fig. 6.2). Within Myrteae, eleven clades were consistently recovered in most of the phylogenetic analyses (Fig. 6.3, 6.6). Obtained clades include the Mediterranean *Myrtus*, another clade only formed by Australasian species and nine other monophyletic groups including South American, Central American and New Zealand species. *Myrtus* was sister to all other Myrteae with weak statistical support in the Bayesian molecular and total evidence analyses (BS 63) and observed in the plastid and molecular strict consensus trees (Table 6.6). The genus *Myrtus* as sister to all other species of Myrteae has been proposed in a number of investigations (Lucas et al., 2007; Murillo et al., 2013, 2016; Thornhill et al., 2015). *Myrtus* was not obtained as sister to the tribe Myrteae in the total evidence analysis using parsimony and was sister to the Australasian group (Fig. 6.5). The sister relationship of *Myrtus* to Australasian species has been suggested in some analyses of Lucas et al. (2007) and two unpublished studies (Costa, 2009; De-Carvalho, 2013). The “Australasian group” (*sensu* Lucas et al., 2007) formed by

Austromyrtus, *Decaspermum*, *Gossia*, *Rhodamnia* and *Rhodomyrtus* was recovered with weak statistical support (BS 50). The sister relationship of *Blepharocalyx salicifolius* to all other Myrteae did not receive bootstrap support, but 0.7 PP in the Bayesian analysis.

Morphological phylogenetic analyses showed weakly supported but well resolved trees (Fig. 6.4). Poorly supported but well resolved trees have been obtained from previous morphological phylogenies in other plant groups (e.g., Magallón, 2007). Morphological strict consensus and 50% majority rule trees recovered similar clades to those obtained in the molecular phylogenetic analyses, supporting a number of genera and relationships. Genera also recovered as monophyletic in morphological analyses include *Amomyrtus*, *Myrceugenia, Luma, Myrcianthes, Eugenia, Myrcia, Calyptranthes, Gossia, Lophomyrtus, Austromyrtus* and *Lenwebbia*. Morphological phylogenetic reconstructions did not recover the Australasian clade and placed *Myrtus* as sister to a clade formed by *Myrceugenia, Luma* and *Blepharocalyx*, but with low support. In the analyses based on morphological data, *Blepharocalyx salicifolius* was not observed as sister to all other Myrteae, but forming a monophyletic group with *Blepharocalyx cruckshanksii* (Fig. 6.1). The close relationship between the two species of *Blepharocalyx* has been obtained in previous molecular phylogenies of Myrteae (Murillo et al., 2013, 2016). Poorly supported relationships in Myrteae using uniquely morphological data have been seen in the studies of Johnson and Briggs (1984) and Wilson et al. (2001).

6.4.2 Phylogenetic relationships of Chilean Myrteae

Chilean species of Myrteae are distributed among six main clades in most of the molecular phylogenetic reconstructions (Figs 6.1, 6.3, 6.6). The phylogenetic position of Chilean genera is fairly consistent among analyses from different methods. As observed in other phylogenetic studies, resolution and statistical support is low in a number of clades and particularly in the spine of the tree (Lucas et al. 2005, 2007; Murillo et al. 2013, 2016). The systematic position of the Chilean genera/species is examined and discussed below.

Amomyrtus + Legrandia

The genus *Amomyrtus* (two species: *A. luma* and *A. meli*) is monophyletic in all analyses (PP 1.0, BS 92). A sister relationship to the Chilean endemic *Legrandia* (one species) is moderately supported in Bayesian and maximum likelihood analyses of molecular data (PP 1.0, BS 70), while there is strong bootstrap support in the total evidence analyses (BS 92)

(Figs 6.1, 6.5, 6.6). The relationship between *Amomyrtus* and *Legrandia* was not observed in the plastid phylogeny. The monophyly of *Amomyrtus* has been suggested by Lucas et al. (2007) but the sister relationship to *Legrandia* was not supported in their analysis. *Amomyrtus meli* and *Legrandia concinna* were sister in Murillo et al. (2013) (PP 0.9), however *Amomyrtus luma* was not included in the former study. A clade containing *Amomyrtus* and *Legrandia* sister to a larger South American clade including *Acca*, *Campomanesia*, *Pimenta* and *Psidium* was obtained in most of the analyses with low statistical support but with a high percentage of times in the consensus trees ("*Pimenta* group", *sensu* Lucas et al., 2007). In the Bayesian phylogeny based on total evidence, the clade *Amomyrtus+Legrandia* was not included in the "*Pimenta* group" but sister to the *Eugenia* group containing *Eugenia* and *Myrcianthes*. The relationship between *Amomyrtus* and *Legrandia* in the "*Pimenta* group" supports previous morphological affinities between the two genera, namely subapical protruding placentas and solitary or racemose inflorescences (McVaugh, 1968; Wilson, 2011).

Blepharocalyx

The genus *Blepharocalyx* is not monophyletic, except for the morphological phylogeny, where it is monophyletic with low support (BS 60) (Fig. 6.4). Generally, the Chilean endemic *Blepharocalyx cruckshanksii* is part of a strongly supported clade (PP 0.98) including *Myrceugenia* and *Luma,* while *Blepharocalyx salicifolius* is placed as an early divergent species of the tribe Myrteae but with low statistical support (BS 57). The clade including *Myrceugenia*, *Blepharocalyx cruckshanksii* and *Luma* is known as the "*Myrceugenia* group" and has been recovered with strong Bayesian support but no bootstrap support in previous studies (Lucas et al., 2007). *Blepharocalyx cruckshanksii* is sister to *Luma* with very low support in the molecular analyses, but with strong bootstrap support in the total evidence analysis using parsimony (BS 92) (Fig. 6.5). Taxonomic revisions have considered *Blepharocalyx cruckshanksii* a "primitive" species in the context of the tribe Myrteae, for having many characters considered plesiomorphic (e.g, C-shaped embryo, dichasial inflorescences) (Kausel, 1947; Landrum, 1988). Other presumably plesiomorphic characters have been described in *Blepharocalyx, Myrceugenia* and *Luma,* suggesting that these genera might be early divergent lineages in Myrteae. However, Lucas et al. (2007) proposed that such characters might be synapomorphies of the "*Myrceugenia* group". It is strongly recommended for future studies to include *Blepharocalyx eggersii* in a phylogenetic sampling to have a more complete hypothesis on the monophyly and systematic position of this genus.

Blepharocalyx cruckshanksii had been previously recognized as a monotypic genus (*Temu* O.Berg). The validity or reestablishment of *Temu* might depend on future molecular evidence that clarifies the phylogenetic relationships of the three species of *Blepharocalyx*.

Luma

The genus *Luma* (2 species, *L. apiculata* and *L. chequen*) is monophyletic in all analyses with strong statistical support (PP 1.0, BS 99-100). In the Bayesian combined molecular analyses, the systematic position of *Luma* was determined as sister to *Blepharocalyx cruckshanksii* with low statistical support. *Luma* + *Blepharocalyx cruckshanksii* was recovered as sister to *Myrceugenia* with strong Bayesian support (PP 0.98).

Myrceugenia

Myrceugenia is strongly supported as monophyletic in all analyses (PP 1.0, BS 98-100) (Fig. 6.3, 6.4, 6.6). Brazilian species of *Myrceugenia* form a distinct clade with strong support (PP 0.95, BS 93) while Chilean species form numerous lineages. The general phylogenetic structure of *Myrceugenia* in this study confirms similar findings from previous studies (Murillo et al. 2012, 2013, 2016). In the morphological and total evidence analyses, Brazilian and Chilean species of *Myrceugenia* have different positions (Figs 6.4, 6.5, 6.6) to those recovered in analyses based only on molecular data. Phylogenetic analyses based on morphological data recovered Brazilian and Chilean species or *Myrceugenia* as part of the same clades, similar to previous morphological phylogenies (Landrum, 1981a). Total evidence analyses have a slightly different topology and improve the statistical support of the groups (Fig. 6.5, 6.6). The Chilean endemic *Myrceugenia rufa* is recovered as sister to all other *Myrceugenia* in most of the analyses and strongly supported in the trees based on nuclear loci (PP 0.98, BS 67) (Table 6.6). In the Bayesian total evidence analysis, a clade formed by *M. rufa, M. correifolia, M. exsucca, M. schulzei, M. colchaguensis* and *M. obtusa* is sister to all other species of *Myrceugenia* with strong Bayesian support (PP 0.95). Within *Myrceugenia*, *Myrceugenia schulzei* (Juan Fernandez Islands) and *Myrceugenia colchaguensis* (endemic to Chile-mainland) form a strongly supported monophyletic group (PP 1.0, BS 100) in the majority of analyses. The two Chilean varieties of *Myrceugenia ovata* (*var. ovata* and *var. nanophylla*) appear monophyletic in the morphological phylogeny (BS 60) (Fig. 6.4). In the molecular analyses, the two Chilean varieties of *Myrceugenia ovata* are not monophyletic and occupy the same phylogenetic position recovered in the study of Murillo et al. (2013) using secondary structure of the nuclear ITS. A strongly supported clade

(PP 1.0, BS 88) includes *Myrceugenia schulzei*, *Myrceugenia colchaguensis*, *Myrceugenia chrysocarpa* and *Myrceugenia obtusa* in the combined molecular analyses (Fig. 6.3). This clade has not been recovered in previous studies. In the Bayesian analysis based on the total evidence data set, a clade including *Myrceugenia rufa*, *Myrceugenia correifolia*, *Myrceugenia exsucca*, *Myrceugenia schulzei*, *Myrceugenia colchaguensis* and *Myrceugenia obtusa* was recovered as sister to all other *Myrceugenia* with strong Bayesian support (Fig 6.6). A similar clade was recovered in Murillo et al. (2016), also sister to all other *Myrceugenia*. *Myrceugenia euosma* (Brazilian) and *Myrceugenia correifolia* (Chilean) appear as monophyletic in the parsimony total evidence tree (Fig. 6.5), possibly due to morphological affinities, since both have xerophytic characteristics. *Nothomyrcia fernandeziana* (previously known as *Myrceugenia fernandeziana*) is confirmed as not part of *Myrceugenia* but more closely related to *Siphoneugena* and *Plinia* (Caribbean and Eastern South America) (PP 1.0, BS 50). Murillo et al. (2013, 2016) placed *Nothomyrcia fernandeziana* as closely related to *Blepharocalyx* with PP 0.8 and no bootstrap support. *Siphoneugena* and *Plinia* were also included in the clade recovered by Murillo et al. (2013) along with *Nothomyrcia* and *Blepharocalyx*.

Myrcianthes

The genus *Myrcianthes* is monophyletic with strong support (PP 0.99, BS 86). The Chilean endemic *Myrcianthes coquimbensis* is recovered as sister to all other species of *Myrcianthes* with strong statistical support (PP 1.0, BS 100) (Figs 6.3, 6.5, 6.6). *Myrcianthes* is closely related to the large genus *Eugenia* with strong support (PP 1.0, BS 100). The "*Eugenia* group" has also been recovered in Murillo et al. (2013). Internal relationships in the *Myrcianthes*-*Eugenia* clade ("*Eugenia* group", *sensu* Lucas et al. 2007) are generally strongly supported in this study and previous investigations (Lucas et al., 2007; Murillo et al., 2013).

Phylogenetic results from this study demonstrated a noticeable difference in the phylogenetic relationships of the genus using two different sampling approaches of *Eugenia*. A first analysis was carried out, including five representatives of *Eugenia* representing the main clades recovered in Mazine et al. (2014). Results indicated that *Eugenia* was embedded in *Myrcianthes* and *Myrcianthes coquimbensis* appeared as sister to *Eugenia* and not included in *Myrcianthes* (results not shown). When all available sequences of *Eugenia* (ca. 70 species) were included in the analyses, *Myrcianthes* and *Eugenia* were recovered as reciprocally monophyletic groups. The final dataset used in this investigation (111 species) with 12

representatives of *Eugenia* recovered the same relationships as the large dataset (179 species) with *Myrcianthes* and *Eugenia* reciprocally monophyletic and *Myrcianthes coquimbensis* sister to all other *Myrcianthes*. This is the first study that investigated the systematic position of *Myrcianthes coquimbensis*. A comprehensive phylogeny of *Myrcianthes* (30 species) with a more robust taxonomic sampling should be conducted to elucidate relationships within the genus and the relationships with *Eugenia*.

Myrteola

The exact systematic position of *Myrteola nummularia* is uncertain in this study. In the Bayesian molecular combined analyses, *Myrteola nummularia* was recovered as part of a clade formed by the New Zealand *Lophomyrtus*, *Neomyrtus*, the Australian *Lenwebbia*, and two *Ugni* species namely *Ugni molinae* and *Ugni myricoides* ("Myrteola group" *sensu* Lucas et al., 2007). The phylogenetic position of *Myrteola nummularia* in this clade was poorly supported in the combined analysis, but had higher support in the plastid phylogenetic analyses (*matK* plus *psbA-trnH*) (PP 0.85). In previous investigations, the phylogenetic position of *Myrteola nummularia* has been indicated in the New Zealand clade (*Lophomyrtus* + *Neomyrtus*) with strong Bayesian support (0.95) but with a poorly supported position in the clade as sister group ("*Myrteola* group") (Lucas et al., 2007). In the parsimony total evidence analysis, *Myrteola nummularia* is recovered as part of a clade formed by *Myrceugenia* and the "*Eugenia* group" (BS 73) and sister to all the other genera in the clade (BS 55). In the Bayesian total evidence analyses *Myrteola* is observed in a clade with *Ugni*, which is sister to the New Zealand-*Lenwebbia* clade (Fig. 6.6.). In Murillo et al. (2013), *Myrteola nummularia* was recovered as part of the "Australasian group" and sister to *Myrceugenia*, both relationships with very low support in two different trees. The relationship between *Myrteola* and New Zealand species has been proposed by Landrum (1988a, 1988b), Lucas et al. (2007) and Murillo et al. (2013). The inclusion of the other two species of *Myrteola*, plus a more robust DNA sampling of *Myrteola nummularia* (widely distributed in South America) might provide a better understanding of the systematic relationships of the genus.

Ugni

Two separate and strongly supported monophyletic groups of *Ugni* were recovered in the genus, namely *Ugni molinae* + *Ugni myricoides* (PP 1.0, BS 100) and *Ugni candollei* + *Ugni selkirkii* (PP 1.0, BS 100) in the majority of analyses. *Ugni molinae* and *Ugni myricoides* were recovered related to the New Zealand genera and to *Myrteola nummularia* (Fig. 6.3) but

with low support. The position of the monophyletic *Ugni candollei* + *Ugni selkirkii* is uncertain in the Bayesian/Parsimony molecular combined reconstructions, as part of a polytomy on the spine of the tree. In the parsimony total evidence analyses, *Ugni candollei* + *Ugni selkirkii* were recovered as sister to all other Myrteae, except for *Blepharocalyx salicifolius*. In the Bayesian total evidence analyses, the two *Ugni* clades are included in a clade with *Myrteola*. The systematic position of *Ugni molinae* has been determined as part of the "*Myrteola* group" along with New Zealand species, with no statistical support (Lucas et al., 2007; Murillo et al. 2013). This is the first attempt to clarify the phylogenetic relationships of *Ugni* including all four recognized species of the genus and supports the non-monophyly of the genus. However, more studies including additional markers are required to elucidate relationships in this genus.

6.5 Conclusions

The molecular and morphological phylogenetic analyses presented here provide new information in the evolutionary context of the tribe Myrteae and particularly Chilean species. Results largely agree with previous phylogenetic studies in Myrteae and particular genera of the tribe (e.g., *Eugenia*, *Myrcia*). Generally, there is not enough statistical support to confirm conflicts with current concepts of genera. A comprehensive morphological data set has provided resolution and improved statistical support for some clades. The use of additional informative loci and morphological characters is recommended for future evolutionary studies in the tribe. A number of taxa should be included to further understand the phylogenetic relationships in Myrteae, such as *Blepharocalyx eggersii, Curitiba prismatica, Amomyrtella guili* and *Calycolpus* spp.

Table 6.1. Primers, sources and PCR amplification conditions used in this investigation

Region	Primers	Sequence (5'-3')	Reference	PCR conditions
ETS	MyrtF	5'-CTCCGTGCTGGTGCATCGAACTGC-3'	Lucas et al. (2007)	3 min at 94ºC followed by 30 cycles of 1 min at 94ºC, 1 min at 50ºC, 1 min at 72ºC
	18S	5'-GAGCCATTCGCAGTTTCACAG-3'	Wright et al. (2001).	
ITS	AB 101	5'-ACGAATTCATGGTCCGGTGAAGTGTTC-3'	Sun et al. (1994).	2 min at 94ºC followed by 30 cycles of 1 min at 94ºC, 1 min at 50ºC, 1 min at 72ºC
	AB 102	5'-GAATTCCCCGGTTCGCTCGCCGTTAC-3'	Sun et al. (1994).	
matK	390F	5'-CGATCTATTCATTCAATATTTC-3'	Johnson and Soltis (1994)	2 min at 94ºC followed by 30 cycles of 1 min at 94ºC, 30 seconds at 46ºC, 1 min at 72ºC
	1326R	5'-TCTAGCACACGAAAGTCGAAGT-3'	Johnson and Soltis (1994)	
psbA-trnH	psbA	5'-CGAAGCTCCATCTACAAATGG-3'	Hamilton (1999)	4 min at 94ºC followed by 30 cycles of 1 min at 94ºC, 1 min at 50ºC, 2 min 30 seconds at 72ºC
	trnH	5'-ACTGCCTTGATCCACTTGGC-3'	Hamilton (1999)	

Table 6.2. Species (authority) and GenBank accession numbers for taxa included in this study. GenBank accession numbers generated in this study highlighted in bold.

Species	Locus			
	ETS	ITS	*psbA-trnH*	*matK*
Acca sellowiana (Berg) Burret	AM489888	AM234067	AM489807	AM489973
Acmena (*Syzygium*) *smithii* (Poir.) Merr. & L. M. Perry	AM489889	AM234137	AM489808	AM489974
Algrizea macrochlamys (DC.) Proença & NicLugh.	AM489890	AM234126	AM489809	AM489975
Amomyrtus luma (Mol.) D.Legr & Kaus.	AM489892	AM234073	AM489811	KM065323
Amomyrtus meli (Phil.) D.Legr. & Kaus.	AM489891	AM234069	AM489810	AM489976
Austromyrtus dulcis (C.T.White) L.S.Sm.	AM489894	AM234133	AM489813	AM489977
Austromyrtus glabra N. Snow & Guymer	**KX509961**	**KX522581**	-	-
Backhousia citriodora F.Muell.	KM064644	KM064810	KC134151	KM065085
Blepharocalyx cruckshanksii (Hook. and Arn.) Nied	AM489895	AM234070	AM489814	AM489978
Blepharocalyx salicifolius (Kunth) O.Berg	AM489896	AM234084	AM489815	AM489979
Calyptranthes concinna DC.	AM489898	AM234103	AM489817	AM489980
Calyptranthes^ kiaerskovii Krug & Urb.	AM489900	AM234105	AM489819	AM489981
Calyptranthes lanceolata O.Berg	AM489899	AM234104	AM489818	-
Campomanesia guazumifolia (Cambess.) O.Berg	AM489902	AM234076	AM489821	AM489982
Decaspermum humile (Sweet ex G. Don) A.J. Scott	AM489905	AM234128	AM489824	AY521534
Eucalyptus perriniana F.Muell. ex Rodway	AM489907	AM234139	AM489825	AM489985
Eucalyptus tetragona (R.Br.) F.Muell.	AM489906	AF190364	AF190381	AM489984
Eugenia arenosa Mattos	KJ187658	KJ187605	KJ469654	-
Eugenia axillaris (Sw.) Willd.	KJ187660	KJ187607	KJ469656	KJ012582
Eugenia bimarginata DC.	KJ187664	KJ187611	KJ469660	-
Eugenia brevistyla D.Legrand	KJ187667	KJ187614	KJ469663	-
Eugenia calycina Cambess.	KJ187669	KJ187616	KJ469665	-

Species	Locus			
	ETS	ITS	*psbA-trnH*	*matK*
Eugenia dysenterica DC.	KJ187672	KJ187620	KJ469669	JX850043
Eugenia nutans O.Berg	KJ187681	KJ187629	KJ469677	-
Eugenia pyriformis Cambess.	AM489914	KJ187639	AM489832	-
Eugenia sulcata Spring ex Mart.	AM489911	AM234089	AM489829	AM489987
Eugenia uniflora O.Berg	AM489910	AM234088	AM489828	AM489986
Gomidesia flagellaris (D.Legrand) Sobral	AM489918	AM234113	AM489836	AM489989
Gomidesia schaueriana O.Berg	AM489917	AM234112	AM489835	AM489988
Gossia hillii (Benth.) N.Snow & Guymer	AM489920	AM234132	AM489838	-
Gossia inophloia (J.Bailey & C.White) Snow and Guymer	AM489919	AM234131	AM489837	-
Hexachlamys hamiltonii Mattos	KJ187653	KJ187653	KJ469703	-
Hexachlamys itararensis Mattos	KJ187707	KJ187654	KJ469704	-
Legrandia concinna (Phil.) Kausel	AM489921	AM234072	AM489839	AM489990
Lenwebbia lasioclada (F. Muell.) N.Snow & Guymer	**KX509962**	**KX522582**	**KX509967**	-
Lenwebbia prominens N.Snow & Guymer	**KX509963**	**KX522583**	KM895153	KM894619
Leptospermum scoparium J.R.Forst. & G.Forst.	AM489922	AM234142	AM489840	AM489991
Lithomyrtus microphylla (Benth.) N.Snow & Guymer	-	**KX522584**	**KX522588**	-
Lophomyrtus bullata Burret	AM489923	AM234145	AM489841	AM489992
Lophomyrtus obcordata (Raoul) Burret	AM489924	AM234146	AM489842	AM489993
Luma apiculata (DC.) Burret	AM489926	AM234101	AM489843	AM489995
Luma chequen (Mol.) A.Gray	AM489927	AM234102	AM489844	JN661010
Marlierea eugeniopsoides (D.Legrand & Kausel) D.Legrand	AM489928	AM234107	AM489845	AM489996
Marlierea obscura O.Berg	AM489930	AM234109	AM489847	AM489997
Marlierea suaveolens Cambess.	AM489929	AM234108	AM489846	-
Metrosideros perforata (J.R.Forst. & G.Forst.) Druce	AM234141	AM234141	AM489848	AM489998
Metrosideros stipularis (Hook. & Arn.) Hook.f.	AM489969	AM234071	AM489884	AF368222

Species	Locus			
	ETS	ITS	*psbA-trnH*	*matK*
Myrceugenia alpigena (DC.) Landrum	AM489937	AM234098	AM489854	JN660991
Myrceugenia alpigena var. fuliginea (O.Berg) Landrum	JN660841	JN660891	-	JN660990
Myrceugenia alpigena var. longifolia (Burret) Landrum	JN660843	JN660893	-	JN660992
Myrceugenia chrysocarpa (O.Berg) Kausel	JN660846	JN660896	**KX522589**	JN660995
Myrceugenia colchaguensis (Phil.) Navas	JN660847	JN660897	**KX522590**	JN660996
Myrceugenia correifolia (Hook. & Arn.) O.Berg	JN660851	JN660901	**KX522591**	JN661000
Myrceugenia euosma (O.Berg) D.Legrand	JN660849	JN660899	-	JN660998
Myrceugenia exsucca (DC.) O.Berg	JN660850	JN660900	**KX522592**	JN660999
Myrceugenia kleinii D.Legrand & Kausel	JN660856	JN660906	-	JN661005
Myrceugenia lanceolata (Juss. ex J. St.-Hil.) Kausel	AM489932	AM234074	AM489849	JN661007
Myrceugenia leptospermoides (DC.) Kausel	AM489933	AM234075	AM489850	AM489999
Myrceugenia myrcioides (Cambess.) O.Berg	JN660865	JN660915	AM489853	AM490000
Myrceugenia obtusa (DC.) O.Berg	JN660866	JN660916	**KX522593**	JN661015
Myrceugenia ovata var. acutata (D.Legrand) Landrum	JN660868	JN660918	-	JN661017
Myrceugenia ovata var. nanophylla (Burret) L.R. Landrum	JN660870	JN660920	**KX522594**	JN661019
Myrceugenia ovata var. ovata (Hook. & Arn.) O.Berg	JN660872	JN660922	**KX522595**	JN661021
Myrceugenia ovata var. regnelliana (O.Berg) Landrum	JN660889	JN660921	-	JN661037
Myrceugenia oxysepala (Burret) D.Legrand & Kausel	JN660873	JN660923	-	JN661022
Myrceugenia parvifolia (DC.) Kausel	JN660874	JN660924	**KX522596**	JN661023
Myrceugenia pinifolia (F. Phil.) Kausel	JN660877	JN660927	**KX522597**	JN661026
Myrceugenia planipes (Hook. & Arn.) O.Berg	AM489934	AM234095	AM489851	JN661027
Myrceugenia reitzii D.Legrand & Kausel	JN660887	JN660937	-	JN661036
Myrceugenia rufa (Colla) Skottsb. ex Kausel	JN660879	JN660929	**KX522598**	JN661028
Myrceugenia schulzei Johow	JN660888	JN660938	**KX522599**	-
Myrcia amazonica DC.	JN091267	JN091215	JN091406	JN091306

Species	Locus			
	ETS	ITS	*psbA-trnH*	*matK*
Myrcia anacardiifolia Gardner	JN091268	JN091216	JN091407	-
Myrcia isaiana G.M.Barroso & Peixoto	JN091281	JN091229	JN091420	JN091311
Myrcia laruotteana Cambess.	AM489939	AM234115	AM489856	AM490002
Myrcia pubescens DC.	JN091285	JN091234	JN091425	-
Myrcia spectabilis DC.	JN091292	JN091241	JN091432	-
Myrcia splendens (Sw.) DC.	JN091294	KF420978	JN091433	JQ588499
Myrcia subverticillaris (O.Berg) Nied.	JN091295	JN091244	JN091435	-
Myrcia tenuivenosa Kiaersk.	JN091297	JN091246	JN091437	JN091317
Myrcia tomentosa (Aubl.) DC.	AM489940	AM234116	AM489857	-
Myrcia torta DC.	JN091298	JN091247	JN091438	JN091318
Myrcianthes cisplatensis (Cambess.) O.Berg	JN660864	JN660914	JQ033349	JN661013
Myrcianthes coquimbensis (Barnéoud) Landrum & Grifo	**KX509964**	**KX522585**	**KX522600**	-
Myrcianthes fragrans (Sw.) McVaugh	KJ772955	KJ772955	KJ772955	KJ772955
Myrcianthes gigantea (D.Legrand) D.Legrand	-	JQ033321	JQ033350	-
Myrcianthes pseudomato (D.Legrand) McVaugh	AM489951	AM234100	AM489868	-
Myrcianthes pungens (O. Berg) D. Legrand	AM489950	AM234099	AM489867	-
Myrteola nummularia (Poir.) O.Berg	AM489954	AM234068	AM489871	AM490008
Myrtus communis L.	AM489955	AM234149	AM489872	AM490009
Neomyrtus pedunculata (Hook. f.) Burret	AM489956	AM234144	AM490637	AM490010
Nothomyrcia fernandeziana (Hook. & Arn.) Kausel	JN660853	JN660903	**KX522601**	JN661002
Pimenta dioica (L.) Merr.	AM489958	KM064833	AM489874	AM490011
Pimenta pseudocaryophyllus (Gomes) Landrum	AM489960	AM234083	AM489876	AM490013
Pimenta racemosa (Mill.) J.W. Moore	AM489959	AM234082	AM489875	AM490012
Plinia pauciflora M.L. Kawas & B. Holst	AM489414	AM489411	AM489570	-
Psidium cattleianum Afzel. ex Sabine	AM489962	AM234080	AM489878	AM490014

Species	Locus			
	ETS	ITS	*psbA-trnH*	*matK*
Psidium cinereum Mart. ex DC.	AM489961	AM234079	AM489877	-
Psidium guajava L.	AY454126	AY487283	HG963647	JQ024986
Rhodamnia argentea Benth.	AM489964	AM234129	AM489880	KM894835
Rhodamnia rubescens Benth.	AM489963	AM234127	AM489879	AM490015
Rhodomyrtus psidioides (G.Don) Benth.	AM489965	AM234134	AM489881	-
Siphoneugena densiflora O.Berg	AM489572	AM489412	AM489571	-
Siphoneugena guilfoyleiana Proença	AM489966	AM234085	AM490638	AM490016
Syzygium jambos (L.) Alston	AM489967	AM234135	AM489882	AM490017
Tristaniopsis laurina (Sm.) Peter G.Wilson & J.T.Waterh.	-	EF041514	-	KM065187
Ugni candollei (Barnéoud) O.Berg	**KX509965**	**KX522586**	**KX522602**	-
Ugni molinae Turcz.	AM489965	AM234134	AM489881	AM489965
Ugni myricoides (Kunth) O.Berg	-	**KX522587**	-	-
Ugni selkirkii (Hook. & Arn.) O.Berg	JN660884	JN660934	**KX522603**	JN661033
Xanthomyrtus compacta Diels	AM489972	AM234148	AM489887	-

Table 6.3. Informative indels scored for each DNA region

Region	Indel	Position
ETS	1	132
	2	135
ITS	3	329-330
	4	765-767
	5	739-744
	6	725-726
	7	729-730
matK	8	258-726
psbA-trnH	9	76-84
	10	85

Table 6.4. Morphological data matrix used in phylogenetic analyses. Characters and character states detailed in Appendix 2.

Taxa	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9
Acca sellowiana	0 0 0 0 0 2 3 0 0 0 ? 0 ? 0 0 0 0 0 0 ? 0 0 2 1 1 0 0 0 0 0 1 2 0 0 0 0 ? ? ? ? 1 0 2 1 1 0 0 1 0 1 2 2 0 1 ? ? 1 1 1 0 1 ? ? 2 ? 0 0 0 0 0 0 0 0 0 ? 1 1 0 1 1
Acmena (*Syzygium*) *smithii* (Poir.) Merr. & L. M. Perry	0 1 0 0 0 0 1 0 0 0 1 0 3 0 0 0 0 0 0 ? 0 0 0 1 1 0 0 0 0 0 0 1 0 1 0 1 0 0 1 0 ? 1 ? ? 0 1 0 2 1 0 2 1 0 0 0 ? ? ? 0 0 ? 0 1 ? 1 0 ? 0 1 0 0 0 0 0 0 0 ? 0 ? 1 1 1
Algrizea macrochlamys (DC.) Proença & NicLugh.	1 1 0 0 0 0 ? 0 0 0 ? 0 ? ? ? ? ? ? ? 0 ? ? 0 ? ? ? ? ? ? ? ? ? ? 1 0 0 0 0 ? 0 ? ? ? ? 2 1 0 1 0 0 2 1 2 2 0 ? 1 0 0 0 ? 0 1 5 ? 2 0 ? 0 ? 0 0 ? ? ? 0 1 1 ? 1 1
Amomyrtus luma (Mol.) D.Legr & Kaus.	0 0 0 0 0 1 ? 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 1 1 0 0 0 1 0 0 0 2 0 0 0 1 0 0 0 ? 0 ? 1 1 0 1 1 0 0 0 0 0 0 0 0 ? 0 0 2 0 1 3 0 2 1 1 0 1 0 1 0 1 1 1 1 1 0 1 0
Amomyrtus meli (Phil.) D.Legr. & Kaus.	0 0 0 0 0 0 ? 0 0 0 1 0 0 0 0 0 0 0 0 0 1 0 0 1 1 0 0 0 1 0 ? 0 2 0 0 0 1 0 0 0 0 0 ? 1 1 0 2 1 0 2 1 0 0 0 0 0 ? 0 0 2 ? 1 3 0 2 1 1 0 1 0 1 0 0 1 1 1 1 0 1 0
Austromyrtus dulcis (C.T.White) L.S.Sm.	1 0 0 1 0 1 ? 0 0 0 0 0 1 0 0 1 1 0 0 0 1 0 1 1 0 0 0 0 1 0 0 1 0 1 1 0 ? 0 0 0 0 ? 2 1 1 1 0 0 0 0 0 0 0 0 ? 0 1 0 0 2 0 1 3 ? 2 0 0 0 1 0 0 0 0 1 1 0 1 0 0 1
Austromyrtus glabra N. Snow & Guymer	1 0 0 0 0 1 ? 0 0 0 0 0 1 0 0 1 1 0 0 0 1 0 1 1 0 0 0 0 1 0 0 1 0 1 1 0 ? 0 0 ? ? ? 2 1 1 2 0 0 1 1 0 0 0 ? 0 1 0 0 2 0 1 3 ? 2 0 ? 0 1 0 0 0 ? ? ? 0 1 0 0 1
Backhousia citriodora F.Muell.	0 1 0 0 0 2 ? 0 0 0 0 1 1 0 0 0 0 0 0 0 0 0 1 1 0 0 0 1 0 0 0 1 0 1 0 0 ? 0 0 ? ? 0 0 0 0 1 0 0 1 1 0 0 0 1 ? ? 0 0 1 0 0 0 2 0 0 ? ? 0 ? 0 0 1 1 0 2 2 1 2 1
Blepharocalyx cruckshanksii (Hook. and Arn.) Nied	0 0 0 0 0 0 ? 0 0 0 1 1 0 0 0 0 0 0 0 0 1 0 0 1 1 0 0 0 1 1 0 1 1 0 1 1 1 0 2 0 1 1 1 1 0 1 2 1 0 2 1 1 1 0 2 0 ? 0 0 1 0 1 2 ? 2 0 0 0 1 0 0 1 0 0 ? 1 0 0 1 0
Blepharocalyx salicifolius (Kunth) O.Berg	0 0 0 0 0 2 ? 0 0 0 ? 1 ? 0 0 0 0 0 0 0 1 0 0 0 1 1 0 0 0 0 ? 0 1 0 1 0 0 0 0 0 1 1 ? 1 0 1 2 1 1 2 1 1 1 0 0 1 0 0 0 1 0 1 2 ? ? 2 0 ? ? ? ? 0 ? ? ? ? 1 1 ? 1 0
Calyptranthes concinna DC.	0 0 0 0 0 1 0 0 1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 1 1 0 0 0 0 0 0 0 1 2 0 0 1 0 0 0 0 ? ? ? 1 0 0 1 1 1 1 1 2 2 0 0 1 0 0 0 0 0 1 0 0 2 0 0 0 1 0 0 1 0 1 0 0 1 0 0 1
Calyptranthes kiaerskovii Krug & Urb.	0 ? 0 0 0 1 ? 0 1 0 ?
Calyptranthes lanceolata O.Berg	0 0 0 0 0 1 0 0 1 0 ? 0 ? ? ? 0 ? ? ? 0 ? ? 0 ? ? ? ? ? 0 ? ? ? ? ? 2 0 0 1 0 0 0 0 ? 0 ? 1 0 0 1 ? 0 1 1 2 2 ? 0 1 ? 0 0 0 0 1 0 0 2 0 0 ? 1 ? ? 1 ? 1 ? 0 1 ? 0 1
Campomanesia guazumifolia (Cambess.) O.Berg	0 0 0 0 0 2 0 0 1 0 0 0 0 0 0 0 0 0 0 ? 0 0 0 1 1 0 ? 1 0 ? 1 0 0 0 0 0 ? 0 0 0 ? ? 1 1 1 0 0 1 0 0 2 2 0 0 0 ? 2 0 1 0 1 2 ? 2 2 ? ? 0 1 ? 0 0 ? ? ? 1 1 ? 1 ?
Decaspermum humile (Sweet ex G. Don) A.J. Scott	0 0 0 0 0 1 ? 0 0 0 1 0 0 0 0 0 0 0 0 0 1 0 0 1 1 0 0 0 1 0 0 1 2 0 0 0 1 ? 0 0 ? ? ? 1 1 1 1 1 1 0 2 2 0 0 0 1 0 1 ? 0 1 0 1 3 ? 2 0 0 0 1 ? 0 ? 1 ? 1 0 1 ? 1 1
Eucalyptus perriniana F.Muell. ex Rodway	0 0 1 0 0 1 3 0 0 0 ? 1 0 0 0 0 0 0 0 0 ? 0 1 0 1 1 0 0 1 0 0 1 1 0 1 0 1 0 0 0 0 1 0 0 0 0 0 0 2 1 0 2 1 2 2 0 ? 0 ? 3 0 ? 1 0 ? 0 1 ? ? ? 1 ? 0 0 0 1 ? 2 2 ? 1 1
Eucalyptus tetragona (R.Br.) F.Muell.	0 0 1 0 0 1 3 0 0 0 ? 0 0 0 1 0 0 0 0 0 0 0 ? 0 1 1 0 ? 1 0 ? 1 1 0 1 0 1 0 0 0 1 0 0 0 0 0 0 2 1 0 2 1 2 2 0 ? 0 ? 2 0 ? 1 0 ? 0 1 0 ? ? 1 0 ? ? ? ? ? 2 1 ? 2 1
Eugenia arenosa Mattos	0 0 0 0 0 0 ? 0 1 0 ?
Eugenia axillaris (Sw.) Willd.	0 ? 0 0 0 1 ? 0 1 0 0 0 0 0 ? 0 0 0 0 0 0 0 ? 1 0 0 ? 0 0 ? 0 2 0 0 1 ? ? 0 0 0 1 0 ? 1 0 2 0 1 2 1 0 0 0 ? 2 1 0 ? ? ? 1 ? ? 2 ? ? ? ? ? ? ? ? ? ? 0 ? 1 ? ? ?
Eugenia bimarginata DC.	0 ? 0 0 0 2 ? 0 1 0 ? 0 ? ? ? ? 0 0 ?
Eugenia brevistyla D.Legrand	0 0 0 0 0 0 ? 0 1 0 ? 0 ? ? ? ? ? 0 ? ? ? ? ? 0 0 ?
Eugenia calycina Cambess.	1 0 0 0 0 2 ? 0 1 0 ? ? ? ? ? ? ? ? ? 0 ?
Eugenia dysenterica DC.	0 ? 0 0 0 2 ? 0 1 0 ? ? 2 0 ? 0 0 0 ? ? 0 0 0 1 0 0 0 0 0 ? 0 2 0 0 1 1 0 0 0 0 ? 1 ? 1 1 0 2 0 1 2 1 0 0 ? ? 0 ? 0 ? 0 0 1 ? ? 2 1 ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
Eugenia nutans O.Berg	1 ? 0 0 0 1 ? 0 1 0 ? ? ? ? 0 ? ? ? ? ? ? 0 ? ? ? ? ? ? ? ? ? ? 0 ? ? ? ? 0 0 ? ? 0 1 1 0 1 0 1 2 1 0 0 ? ? 0 1 ? ? ? ? 1 1 1 ? 1 ? ? ? ? ? ? ? ? ? ? ? ? ? ?
Eugenia pyriformis Cambess.	0 0 0 0 0 1 0 0 1 0 ? ? ? 0 0 0 0 0 0 ? 0 0 0 1 0 0 0 0 0 0 ? 0 2 0 0 1 1 0 0 0 0 1 0 1 0 1 0 0 1 1 1 ? ? 0 2 ? ? 0 0 0 0 1 ? 1 2 1 ? ? ? ? 0 ? ? ? 0 1 1 ? 1 0
Eugenia sulcata Spring ex Mart.	0 0 0 0 0 0 0 0 1 0 1 0 2 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 2 0 0 1 1 0 0 0 ? 1 0 ? ? ? ? ? ? ? ? ? 0 0 ? 2 0 1 0 0 0 0 1 1 ? 2 1 ? ? 0 0 0 0 0 1 1 ? ? 1 ? 1 0
Eugenia uniflora O.Berg	0 0 0 0 0 2 0 0 1 0 0 0 2 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 ? 0 0 0 2 0 0 1 1 0 0 0 ? 1 0 1 1 0 2 0 1 2 1 0 0 0 2 0 1 0 0 0 0 1 1 2 2 1 ? 0 1 0 0 0 0 1 1 0 0 1 0 1 0
Gomidesia flagellaris (D.Legrand) Sobral	0 0 0 0 0 1 0 0 0 0 ? 0 1 ? 0 ? ? ? 0 ? ? ? ? ? ? ? ? ? ? 0 ? ? ? ? 0 1 0 1 ? 0 0 0 ? ? ? 1 1 0 1 1 0 1 1 2 2 ? ? 1 ? 1 0 0 0 1 0 ? 2 0 ? ? ? ? ? ? ? ? ? 0 0 1 ? 1 1
Gomidesia schaueriana O.Berg	0 0 0 0 0 1 ? 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 1 1 0 2 0 0 ? 0 0 0 ? ? 1 1 1 1 0 0 1 1 0 0 0 ? 0 1 0 0 2 0 1 3 ? 2 0 ? ? 1 ? 0 ? ? ? 1 1 1 0 1 1
Gossia hillii (Benth.) N.Snow & Guymer	1 0 0 0 0 1 ? 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 ? 1 1 0 2 0 0 ? 0 0 ? ? ? 1 1 1 1 0 0 1 1 0 0 0 ? 0 1 0 0 2 0 1 3 ? 2 0 ? 0 1 ? ? 0 0 0 ? 1 1 ? 1 1
Gossia inophloia (J.B and C.W) Snow and Guymer	0 0 0 0 0 1 ? 0 0 0 ? 0 1 ? 0 1 ? ? ? ? 0 0 ? ? ? ? ? ? ? ? ? ? ? ? ? 2 2 0 ? ? ? ? ? ? ? ? ? 1 ? ? 2 0 ? ? ? ? ? ? ? ? ? ? 0 ? ? ? ?
Hexachlamys hamiltonii Mattos	0 0 0 0 0 1 ? 0 0 0 ? 0 ? ? ? ? ? ? ? 0 ? ? 0 ? ? ? ? ? 0 ? ? ? 0 ? ? 1 0 ? 1 ? ? 0 0 0 ? ? ? 1 0 2 1 0 1 1 0 0 ? 0 0 ? 0 0 1 ? ? ? ? ? ? ? ? ? 0 ? ? ? ? 0 ? 1 ? 1 0
Hexachlamys itararensis Mattos	0 ? 0 0 0 1 ? 0 0 0 ? ? ? ? ? 1 1 0 0 ? 0 0 0 ? ? 0 ? 0 0 0 ? 0 1 1 1 ? 1 ? 0 0 ? ? ? ? 1 0 2 1 0 1 1 0 0 ? 0 ? ? 0 0 ? ? 1 ? ? 2 ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? 0
Legrandia concinna (Phil.) Kausel	0 0 0 0 0 1 ? 0 0 0 1 0 0 0 0 0 0 0 0 0 1 1 ? 1 1 0 0 0 1 0 0 0 0 0 0 0 0 0 ? 0 0 0 0 ? 2 1 1 1 0 1 1 1 0 0 0 0 0 ? 0 0 2 0 1 2 1 2 0 0 0 1 0 0 0 1 1 1 1 1 1 0 0
Lenwebbia lasioclada (F. Muell.) N.Snow & Guymer	0 1 0 0 0 1 ? 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 1 0 0 0 1 0 0 0 0 1 0 0 0 0 1 0 0 0 0 0 0 0 0 ? 2 1 1 0 0 1 0 1 0 0 0 1 0 ? 1 0 ? 0 1 3 1 2 0 ? 0 1 0 0 0 1 1 1 0 0 0 1 0
Lenwebbia prominens N.Snow & Guymer	0 0 0 0 0 1 ? 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 1 1 0 0 0 1 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 ? 2 1 1 0 0 1 0 1 0 0 0 ? 0 ? 1 0 ? 0 1 3 ? 2 0 0 0 1 ? 0 0 ? ? ? 0 0 ? 1 0
Leptospermum scoparium J.R.Forst. & G.Forst.	0 0 1 0 0 ? 0 0 0 0 ? 0 0 1 1 0 0 0 0 ? 0 1 1 ? 0 0 0 0 0 0 0 0 2 0 1 0 0 ? 0 0 1 ? ? 0 1 1 2 0 0 1 1 ? ? ? ? 1 ? ? 0 ? 0 0 ? 2 ? ? ? ? 1 0 0 0 0 ? ? 1 2 1 1 2 1
Lithomyrtus microphylla (Benth.) N.Snow & Guymer	1 0 0 1 0 0 ? 0 0 0 1 0 0 1 0 0 0 0 0 2 1 1 1 1 0 1 0 1 1 0 0 2 0 0 0 0 ? 0 0 0 ? ? 2 1 1 0 0 0 0 0 0 0 0 0 ? 0 ? 4 0 ? 0 1 3 ? 2 0 ? ? 1 0 ? 1 0 0 ? 2 0 0 0 0
Lophomyrtus bullata Burret	0 0 0 0 0 0 ? 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 1 0 0 1 1 0 0 0 1 0 0 0 0 0 0 1 0 2 0 1 1 ? 2 1 1 2 0 1 2 1 0 0 0 1 0 ? 0 0 2 0 1 3 ? 2 0 0 ? 1 0 1 0 1 1 1 0 0 ? 0 0
Lophomyrtus obcordata (Raoul) Burret	0 0 0 0 0 0 ? 0 0 0 0 1 0 1 0 0 0 0 0 0 0 0 1 0 0 1 1 0 0 0 1 0 0 0 0 0 0 0 0 0 1 0 2 0 1 1 ? 2 1 1 2 0 1 2 1 0 0 ? 3 0 ? 0 0 2 0 1 3 ? 2 0 ? ? 1 ? 1 0 ? ? ? 0 0 ? 0 0
Luma apiculata (DC.) Burret	0 0 0 0 0 0 ? 0 0 0 1 0 2 0 0 0 0 0 0 0 1 0 0 1 1 0 0 0 1 0 0 0 2 0 2 0 0 0 1 0 1 0 ? 1 1 0 1 1 1 1 1 1 1 0 0 0 ? 0 0 1 0 1 4 1 2 0 0 0 1 0 0 1 0 0 ? 1 1 0 1 0
Luma chequen (Mol.) A.Gray	0 0 0 0 0 0 ? 0 0 1 1 0 2 0 0 0 0 0 0 0 0 0 1 1 0 0 0 1 1 0 1 2 0 1 0 0 ? 1 0 1 0 ? 1 1 0 2 0 1 2 1 1 1 0 0 0 ? 0 0 1 0 1 4 1 2 0 0 0 1 0 0 1 0 0 ? 0 0 0 1 0
Marlierea eugeniopsoides (D.Legrand & Kausel) D.Leg.	0 0 0 0 0 1 ? 0 0 0 ? ? 2 ? ? ? ? ? ? ? ? ? ? 0 ? ? ? 0 0 ? ? 1 1 0 ? ? ? ? 0 ? ? ? ? ? 1 0 ? ? ? ? ? 2 2 ? ? 1 ? ? 0 ? ? 1 0 ? 2 0 ? ? 1 ? 0 ? ? ? ? 0 ? ? ? ?
Marlierea obscura O.Berg	0 0 0 0 0 1 ? 0 1 0 0 0 2 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 1 0 0 1 1 0 0 ? ? 0 ? 0 1 0 1 1 0 1 1 2 2 ? 0 ? ? 0 0 0 0 0 1 0 ? 2 0 0 ? ? ? 0 ? 0 0 0 0 1 ? 1 1
Marlierea suaveolens Cambess.	0 ? 0 0 0 1 ? 0 0 0 ? ? 1 0 ? 0 0 0 0 ? 0 ? 0 1 1 0 0 ? 0 ? 1 1 0 1 1 1 0 0 ? ? 0 ? 0 1 0 1 1 0 1 1 2 2 ? ? ? ? 1 ? 0 ? 1 0 ? 2 0 ? ? ? ? ? ? ? ? ? ? ? ? 1 1
Metrosideros perforata (J.R.Forst. & G.Forst.) Druce	0 0 0 0 0 0 ? 0 0 0 1 0 0 0 0 0 0 0 0 1 2 1 0 1 1 0 1 1 1 2 0 0 1 1 0 0 1 0 0 0 0 1 1 1 0 1 1 1 0 0 2 1 ? ? 0 1 1 ? 1 0 ? 0 0 4 0 0 ? 0 1 1 0 1 ? 1 1 0 2 0 ? 1 1
Metrosideros stipularis (Hook. & Arn.) Hook.f.	0 0 0 0 0 0 ? 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 1 0 0 0 2 1 1 1 0 0 2 0 1 1 1 2 1 1 2 0 0 2 1 0 0 1 3 1 ? 1 0 ? 0 0 4 0 0 0 ? 1 1 ? 1 0 1 1 0 0 1 ? 1 1

Table 6.4 continuation.

	1 2 3 4 5 6 7
Taxa	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9
Myrceugenia alpigena (DC.) Landrum	0 ? 0 1 0 1 ? 0 1 0 ? 0 ? ? ? ? ? ? 0 ? ? ? ? ? ? ? ? ? ? 0 ? 0 0 1 0 0 ? 2 1 ? ? ? 2 1 0 0 0 1 1 1 1 1 0 ? ? ? 0 ? ? ? 1 ? ? ? ? ? ? ? ? ? ? ? ? ? ? 1 ? 1 1
Myrceugenia alpigena var. fuliginea (O.Berg) Landrum	0 0 0 1 0 1 ? 0 1 0 ? ? ? ? ? ? ? ? ? ? ? 0 ? ? ? ? ? ? ? ? ? ? 0 ? ? ? ? 0 ? ? ? ? ? 1 0 0 0 1 1 1 1 1 0 ? ? ? 0 ? ? ? 1 0 ? ? ? ? ? ? ? 0 ? ? ? ? ? ? ? ? ?
Myrceugenia alpigena var. longifolia (Burret) Landrum	0 ? 0 1 0 1 ? 0 1 0 ? 0 ? ? ? ? 0 ? ? ? ? ? 1 0 0 0 1 1 1 1 1 ? ? 0 ? ? ? ? ? 1 0 ? 2 ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
Myrceugenia chrysocarpa (O.Berg) Kausel	1 0 0 0 0 1 ? 1 1 0 1 0 0 0 0 0 0 0 0 0 0 0 1 1 2 0 0 0 0 ? 0 0 0 2 0 0 ? 2 1 0 ? ? 2 1 1 1 0 1 2 1 1 1 ? 0 0 1 0 0 1 0 1 0 ? 2 0 ? 0 1 ? 0 1 1 1 1 0 0 0 0 1
Myrceugenia colchaguensis (Phil.) Navas	1 0 0 1 0 0 ? 0 1 0 1 0 5 0 0 0 0 0 0 2 0 0 1 1 1 0 0 0 0 0 0 0 0 1 0 0 ? 0 1 0 ? ? 2 1 1 0 0 1 0 0 1 1 0 ? 0 1 0 ? 1 0 1 0 ? 2 0 ? 0 ? ? ? 0 1 1 ? 2 0 0 1 1
Myrceugenia correifolia (Hook. and Arn.) O.Berg	0 0 0 1 0 1 ? 1 1 0 1 0 0 0 0 0 0 0 0 2 1 0 1 1 0 0 0 0 1 0 0 0 0 1 0 0 ? 2 0 0 ? ? 2 1 1 0 0 0 0 0 1 1 0 ? 0 1 0 0 1 0 1 0 ? 2 0 ? 0 1 0 0 1 1 1 1 2 0 0 1 1
Myrceugenia euosma (O.Berg) D.Legrand	0 ? 0 1 0 0 ? 0 1 1 ? ? ? 1 0 0 0 1 0 ? 1 0 2 1 1 0 0 0 0 0 1 0 0 ? ? ? ? 2 1 ? ? ? ? 0 1 0 0 1 0 0 1 1 0 ? ? ? ? ? ? ? 1 ? ? 2 0 ? ? ? ? 0 ? ? ? 1 ? 0 ? 0 1
Myrceugenia exsucca (DC.) O.Berg	0 0 0 1 0 1 ? 1 1 0 0 1 0 0 0 0 0 0 0 0 0 0 1 1 0 0 0 1 0 0 0 0 0 1 0 1 ? 0 1 0 ? ? 0 0 0 0 0 1 0 0 1 1 0 0 ? 1 0 0 1 0 1 ? ? 2 0 1 0 1 0 0 1 1 1 1 0 1 0 0 1
Myrceugenia kleinii D.Legrand & Kausel	0 ? 0 0 0 1 ? 0 1 0 ? ? 0 ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? 0 ? ? ? ? ? ? ? ? ? ? 1 0 1 1 1 1 1 1 1 ? ? ? ? ? ? ? 0 1 0 ? 2 ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
Myrceugenia lanceolata (Juss. ex J. St.-Hil.) Kausel	1 0 0 0 0 1 ? 0 1 0 1 0 0 0 0 0 0 0 0 0 0 0 1 1 0 0 ? 1 0 0 0 0 0 2 0 1 ? 2 1 ? 0 ? 2 0 1 1 0 1 1 1 1 1 0 0 0 1 0 0 1 ? 1 0 1 2 0 ? 0 1 ? ? 0 1 1 ? 0 0 0 1 1
Myrceugenia leptospermoides (DC.) Kausel	1 0 0 0 0 0 ? 0 1 0 1 0 0 0 0 0 0 0 0 0 0 0 1 1 0 0 0 1 0 0 0 0 0 1 0 0 ? 2 0 ? ? ? 2 1 1 1 0 1 2 1 1 1 0 0 0 ? ? 0 1 0 1 ? ? 2 0 ? 0 1 0 0 0 1 1 ? 0 1 0 1 1
Myrceugenia myrcioides (Cambess.) O.Berg	0 ? 0 0 0 1 ? 0 1 0 ? ? ? ? 0 ? ? ? 0 ? ? 0 ? ? ? ? ? ? ? 0 ? ? 0 1 ? ? ? 2 1 ? ? ? 2 1 1 0 0 1 0 0 1 1 ? ? ? ? ? 0 ? 0 1 ? ? 2 ? ? ? ? ? 0 ? ? ? ? 0 1 ? 1 1
Myrceugenia obtusa (DC.) O.Berg	0 0 0 0 0 1 ? 0 1 1 0 0 0 0 0 0 0 0 0 0 0 0 1 1 0 0 0 1 1 0 0 0 0 2 0 0 ? 2 ? ? 0 ? 2 1 1 1 0 1 2 1 1 1 0 ? 0 1 0 0 1 0 1 0 ? 2 0 0 0 1 0 0 0 ? ? ? 0 1 0 0 1
Myrceugenia ovata var. acutata (D.Legrand) Landrum	0 ? 0 0 0 0 ? 0 1 0 ? ? ? ? ? 0 0 ? ? ? ? ? 1 ? 1 0 ? 0 0 ? 0 ? 0 ? 0 ? ? 2 1 ? ? ? 2 1 1 1 0 1 2 1 1 1 ? ? ? ? ? 0 ? 0 1 0 1 ? ? ? ? ? ? ? ? ? ? ? 0 ? ? ? ?
Myrceugenia ovata var. nanophylla (Burret) L.R. Landrum	0 0 0 0 0 0 ? 0 1 0 1 1 0 0 0 0 0 0 0 0 0 0 1 1 0 0 0 1 0 0 0 0 0 2 0 1 ? 1 ? 0 ? ? 2 1 1 1 0 1 1 1 1 1 0 ? ? ? ? ? 1 0 1 0 ? 2 0 0 0 1 ? 0 0 1 1 ? 0 0 ? 1 1
Myrceugenia ovata var. ovata (Hook. and Arn.) O.Berg	0 0 0 0 0 0 ? 0 1 0 1 1 0 0 0 0 0 0 0 0 0 0 1 1 0 0 0 1 1 0 0 0 0 2 0 1 ? 2 1 ? ? ? 2 1 1 1 0 1 1 1 1 1 0 ? ? ? ? ? 1 0 1 0 ? 2 0 0 0 1 ? 0 0 1 1 ? 0 0 0 0 1
Myrceugenia ovata var. regnelliana (O.Berg) Landrum	0 ? 0 0 0 0 ? 0 1 0 ? ? 0 ? ? 0 ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? 0 ? ? ? ? 2 1 ? ? ? 2 1 1 1 0 1 2 1 1 1 ? ? ? ? ? ? ? ? 1 ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? 0 ? ?
Myrceugenia oxysepala (Burret) D.Legrand & Kausel	0 ? 0 0 0 1 ? 0 1 0 ? ? 0 ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? 0 ? ? ? ? 2 1 ? ? ? ? 1 1 0 0 1 2 1 1 1 ? ? ? ? ? ? ? 0 1 ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
Myrceugenia parvifolia (DC.) Kausel	0 0 0 0 0 1 ? 0 1 0 1 0 0 0 0 1 1 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 1 0 0 ? 2 1 ? 0 ? ? 1 1 1 0 1 2 1 1 1 0 ? ? ? 0 0 1 0 1 0 1 2 0 0 0 0 ? 0 0 1 1 ? 0 0 0 1 1
Myrceugenia pinifolia (F. Phil.) Kausel	1 0 0 0 0 0 ? 0 1 0 1 0 0 0 0 0 0 0 0 1 0 0 1 1 0 0 0 0 0 0 0 0 0 2 0 1 ? 2 1 0 0 ? 2 1 1 1 0 1 2 1 1 1 0 ? ? ? ? ? 1 0 1 0 1 2 0 ? 0 1 ? 0 0 1 1 ? 1 1 0 1 1
Myrceugenia planipes (Hook. and Arn.) O.Berg	0 0 0 0 0 1 ? 1 1 0 1 0 0 0 0 0 0 0 0 0 0 0 1 1 2 0 0 0 0 0 0 0 0 2 0 0 ? 2 1 0 0 ? 2 1 0 1 0 1 2 1 1 1 0 ? ? 1 0 0 1 0 1 0 ? 2 0 0 0 1 ? 0 0 1 1 1 0 0 0 1 1
Myrceugenia reitzii D.Legrand & Kausel	0 ? 0 0 0 1 ? 0 1 0 ? ? 0 ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? 0 ? ? ? ? ? ? ? ? ? ? 1 0 0 0 1 ? ? 1 1 ? ? ? ? ? 0 1 0 1 0 ? 2 ? ? ? ? ? ? ? ? ? ? ? ? ? 1 ?
Myrceugenia rufa (Colla) Skottsb. ex Kausel	1 0 0 1 0 0 ? 1 1 0 1 0 0 1 0 0 0 0 1 2 0 0 2 1 0 0 0 1 0 0 0 0 0 0 0 0 0 2 1 0 ? ? 2 1 0 0 0 1 0 0 1 1 0 0 0 1 ? 0 1 0 1 0 2 2 0 0 0 1 0 0 0 1 1 1 2 1 0 1 1
Myrceugenia schulzei Johow	0 0 0 1 0 1 ? 1 1 0 1 0 0 0 0 0 0 0 0 0 1 0 1 0 1 0 0 0 0 0 0 0 0 2 0 0 ? 2 1 0 ? ? 1 0 1 0 0 1 0 0 1 1 ? 0 0 ? ? 0 1 0 1 0 1 2 0 0 0 1 0 ? ? 1 ? ? 0 0 0 1 1
Myrcia amazonica DC.	0 ? 0 0 0 1 ? 0 1 0 ? 0 0 ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? 0 ? ? ? ? ? 0 ? ? ? 0 1 0 1 1 0 1 1 2 2 ? ? ? ? 0 ? 0 0 1 ? ? 2 0 ? ? ? ? 0 ? ? ? ? ? ? ? ? ?
Myrcia anacardiifolia Gardner	0 ? 0 0 0 1 ? 0 0 0 ? ? 0 ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? 0 ? ? ? ? ? 0 ? ? ? ? 1 0 1 1 0 1 1 2 2 ? ? 1 ? ? ? ? ? 1 0 1 2 ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
Myrcia isaiana G.M.Barroso & Peixoto	0 ? 0 0 0 1 ? 0 1 0 ? 0 0 ? ? ? ? ? 0 ? ? ? ? ? ? ? ? ? ? 0 ? 1 0 1 1 1 ? ? 0 ? ? ? ? ? ? ? ? ? ? ? 2 2 0 ? 1 ? 0 0 0 0 1 0 1 2 0 ? 0 0 ? 0 ? ? ? 0 ? ? ? ? ?
Myrcia laruotteana Cambess.	0 0 0 0 0 1 0 0 1 0 1 0 2 0 0 0 0 0 0 0 0 0 1 1 0 0 0 0 0 0 0 1 1 1 1 1 0 ? 0 ? ? ? 0 1 0 1 1 0 1 1 2 2 ? 0 1 0 1 0 0 0 1 0 1 2 0 ? ? 0 ? 0 0 ? ? 0 1 1 ? 1 1
Myrcia pubescens DC.	0 ? 0 0 0 1 ? 0 1 0 1 ? 2 0 ? 0 0 0 ? 0 0 ? 1 1 0 0 ? 0 0 ? 0 ? 0 ? 1 ? 0 ? 0 0 ? ? ? 1 0 0 1 1 0 0 2 2 ? ? ? ? ? ? ? 0 1 0 ? 2 0 ? ? 0 ? ? ? ? ? ? ? ? ? 1 1
Myrcia spectabilis DC.	0 ? 0 0 0 1 ? 0 0 0 ? ? 0 ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? 0 ? ? 1 ? ? 0 0 ? ? 0 1 0 1 1 1 1 1 2 2 ? ? ? ? 1 ? ? 0 1 ? ? 2 ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
Myrcia splendens (Sw.) DC.	0 ? 0 0 0 1 ? 0 1 0 ? ? 0 ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? 0 1 ? 1 ? ? 0 ? ? ? ? 1 0 1 1 1 1 1 2 2 ? ? ? ? ? ? ? 0 1 ? ? 2 0 0 ? 0 ? ? ? ? ? ? ? ? ? ? ?
Myrcia subverticillaris (O.Berg) Nied.	0 ? 0 0 0 1 ? 0 0 0 ? ? 0 ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? 1 0 ? ? ? ? ? ? 0 ? ? 0 ? ? ? ? ? ? ? 2 2 ? ? ? ? 2 ? ? ? 1 0 ? ? 0 ? ? ? ? ? ? ? ? ? ? ? ? ? ?
Myrcia tenuivenosa Kiaersk.	0 ? 0 0 0 1 ? 0 0 0 ? 0 0 ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? 1 ? 1 ? 0 ? ? ? ? ? 0 1 0 1 1 1 1 1 2 2 ? ? 1 ? ? ? ? ? 1 ? ? 2 0 ? ? ? ? ? ? ? ? ? ? ? ? ? ?
Myrcia tomentosa (Aubl.) DC.	0 0 0 1 0 2 0 0 1 0 1 0 0 0 0 0 ? 0 0 2 0 0 1 1 0 0 0 0 0 0 0 1 1 1 1 1 0 ? 0 ? ? ? 0 1 0 0 1 1 0 0 2 2 ? 0 ? 0 2 0 0 0 1 0 1 2 0 0 0 0 0 0 0 0 0 0 2 1 0 1 1
Myrcia torta DC.	0 0 0 0 0 1 0 0 0 0 ? 0 0 0 0 0 ? 0 0 0 0 0 1 1 0 0 0 0 0 0 0 1 0 1 1 0 0 ? 0 ? ? ? 0 1 0 0 1 1 ? ? 2 2 ? 0 ? 0 ? 1 0 0 1 0 ? 2 0 ? 0 ? 0 0 ? ? ? 0 0 1 ? 1 1
Myrcianthes cisplatensis (Cambess.) O.Berg	0 0 0 0 0 1 ? 0 0 0 ? ? 0 ? ? ? ? ? 0 ? ? 0 ? ? ? ? 0 ? ? 0 0 ? 0 0 1 ? 0 ? ? ? 0 ? 2 0 1 2 0 0 2 1 0 0 0 ? 0 ? 0 ? ? ? 1 ? ? 2 1 0 0 ? ? ? ? ? ? ? 0 1 ? 1 1
Myrcianthes coquimbensis (Barnéoud) Landrum & Grifo	1 0 0 1 0 1 ? 0 0 1 1 1 4 0 0 2 0 0 0 0 0 0 1 1 0 0 0 1 0 0 0 2 0 1 1 0 0 0 0 0 1 ? 2 1 0 0 0 0 0 0 0 0 0 2 0 ? 0 0 2 ? 1 1 2 2 1 1 1 1 0 1 0 0 0 0 2 0 0 1 0
Myrcianthes fragrans (Sw.) McVaugh	0 0 0 0 0 1 ? 0 0 0 ? 0 0 ? 0 2 0 1 0 0 0 0 1 1 0 1 0 0 0 0 0 1 0 0 1 1 0 0 ? 0 ? ? 2 0 1 2 0 1 1 1 0 0 0 2 0 ? 0 ? ? 0 1 1 ? 2 1 0 ? 1 0 ? ? ? ? 0 0 0 ? 1 1
Myrcianthes gigantea (D.Legrand) D.Legrand	0 0 0 0 0 1 ? 0 0 0 0 0 0 0 0 ? ? 0 0 0 0 0 1 1 0 0 0 0 0 ? 0 1 0 0 1 1 0 ? 0 0 ? ? 2 0 1 2 0 1 2 1 0 0 ? 2 0 ? 0 0 2 0 1 1 ? 2 1 ? ? ? ? ? ? ? ? ? 0 1 ? 1 1
Myrcianthes pseudomato (D.Legrand) McVaugh	0 0 0 0 0 1 ? 0 0 0 0 0 0 0 0 ? ? 0 0 0 0 0 0 1 0 1 0 0 0 0 0 1 0 0 1 1 0 ? 0 ? ? ? 2 1 0 2 0 1 2 1 0 0 ? ? ? ? 0 0 2 ? 1 1 ? 2 1 ? ? ? ? ? ? ? ? 0 1 0 0 1 1
Myrcianthes pungens (O. Berg) D. Legrand	0 0 0 0 0 1 ? 0 0 0 1 0 0 0 0 0 0 1 0 0 1 0 0 1 0 1 0 0 0 0 0 1 0 0 1 1 0 0 ? 0 0 ? 2 0 1 2 0 1 2 1 0 0 0 ? 0 ? 0 ? 2 0 1 1 ? 2 1 0 0 0 0 ? ? ? ? 0 1 0 0 1 1
Myrteola nummularia (Poir.) O.Berg	2 0 0 0 0 0 ? 0 0 0 0 0 0 0 1 1 1 0 0 0 0 0 1 1 0 0 0 1 1 0 0 2 0 0 0 0 ? 1 0 ? 0 1 2 1 1 2 0 0 2 1 0 0 1 0 0 1 0 0 2 0 1 3 ? 2 0 1 0 1 0 ? 1 0 1 1 0 1 0 0 1
Myrtus communis L.	0 0 0 0 0 1 ? 0 0 0 ? 0 ? ? 0 ? ? ? 0 0 ? 0 ? ? ? ? 0 ? ? 0 ? 1 0 0 1 0 ? 0 1 1 ? ? 2 1 1 2 0 0 2 1 0 0 0 0 0 1 1 0 1 0 1 3 ? 2 0 ? 0 1 0 ? 1 1 1 1 0 1 0 1 1
Neomyrtus pedunculata (Hook. f.) Burret	0 0 0 0 0 1 ? 0 0 0 ? 0 ? ? 0 0 0 0 0 0 1 0 1 1 1 0 0 0 0 0 0 0 0 0 0 1 0 1 0 1 1 ? 2 1 1 2 0 0 2 1 0 0 ? 0 ? 1 4 0 2 0 1 3 ? 2 0 0 ? 1 0 1 1 0 1 1 0 0 ? 0 0
Nothomyrcia fernandeziana (Hook. & Arn.) Kausel	0 0 0 0 0 2 ? 0 0 0 0 1 0 0 0 0 0 1 0 0 0 0 1 1 0 0 0 1 0 0 1 1 0 2 1 0 ? 0 0 ? 1 ? 0 1 0 0 1 0 1 1 0 0 ? ? ? ? 0 0 ? 0 1 ? 1 2 0 0 0 1 ? 0 1 0 1 ? 0 1 0 0 1

Table 6.4 continuation.

	1										2										3										4										5										6										7								
Taxa	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9
***Pimenta dioica* (L.) Merr.**	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	?	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	1	1	0	1	1	?	?	?	?	1	?	1	0	1	2	1	1	2	1	0	0	0	0	0	1	0	0	2	?	1	2	1	2	1	?	?	?	?	?	?	?	1	0	0	?	1	1
***Pimenta pseudocaryophyllus* (Gomes) Landrum**	0	?	0	0	0	2	?	0	0	0	?	?	0	0	?	1	1	1	0	0	0	0	1	1	1	0	?	1	0	?	1	1	1	0	1	1	?	?	0	0	?	?	1	0	1	2	1	1	2	1	0	0	?	0	0	2	0	0	2	0	1	2	1	2	1	?	?	?	?	0	?	?	?	?	?	0	?	?	1
***Pimenta racemosa* (Mill.) J.W. Moore**	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	2	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	1	1	0	1	1	0	0	0	0	?	?	1	0	1	2	1	1	2	1	0	0	?	0	0	2	0	0	2	?	1	2	1	2	1	?	0	0	?	0	0	1	1	1	0	0	0	1	1
***Plinia pauciflora* M.L. Kawas & B. Holst**	0	?	0	0	0	1	?	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	1	0	1	1	1	0	0	0	0	0	?	0	1	0	1	1	1	1	1	2	2	?	?	?	1	0	?	0	0	1	1	?	?	0	?	?	?	?	?	?	?	?	0	0	1	?	1	1
***Psidium cattleianum* Afzel. ex Sabine**	0	0	0	0	0	1	0	0	0	0	?	?	2	0	?	1	1	0	0	2	0	0	1	1	1	0	?	0	0	?	0	0	1	0	1	1	?	0	?	?	0	?	1	1	0	2	1	0	2	1	0	0	0	0	?	?	0	1	2	0	1	3	?	2	1	?	?	?	?	0	?	?	?	?	0	1	?	?	1
***Psidium cinereum* Mart. ex DC.**	0	0	0	0	0	2	0	0	0	0	1	?	2	0	?	1	1	0	0	2	0	?	1	1	1	0	?	0	0	0	0	0	0	1	0	1	1	?	0	0	0	?	?	2	0	1	2	1	0	2	1	0	0	0	0	?	?	0	1	2	0	1	3	1	?	1	?	?	?	?	0	?	?	?	?	1	0	1	?	1	1
***Psidium guajava* L.**	0	?	0	0	0	2	0	0	0	0	1	0	2	0	0	1	1	1	0	0	0	0	1	1	1	0	0	1	0	0	1	0	1	0	1	1	0	0	0	1	?	?	2	1	1	2	0	0	2	1	0	0	?	0	?	?	0	1	2	0	1	3	1	2	1	0	0	0	1	?	0	1	1	1	2	1	?	1	1
***Rhodamnia argentea* Benth.**	0	0	0	0	0	1	2	?	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0	0	0	1	0	?	1	1	0	0	0	1	0	0	0	?	1	0	1	1	1	1	1	1	1	1	0	0	?	0	0	1	4	0	2	0	1	3	0	?	0	0	?	1	?	0	1	1	1	1	1	?	0	1
***Rhodamnia rubescens* Benth.**	0	0	0	0	1	2	?	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0	1	0	1	0	0	1	1	0	0	0	1	0	0	?	0	1	0	1	1	1	1	1	1	1	1	1	0	0	?	0	0	1	4	0	2	0	?	1	3	0	2	0	?	0	1	?	0	1	1	1	1	1	1	0	1
***Rhodomyrtus psidioides* (G.Don) Benth.**	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0	1	0	0	0	0	1	1	0	0	0	1	0	0	0	0	?	?	1	0	1	0	1	0	0	0	0	0	?	0	0	1	1	0	1	?	1	3	1	2	0	0	0	1	?	?	0	1	1	1	0	0	1	0	1
***Siphoneugena densiflora* O.Berg**	0	0	0	0	0	1	?	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	1	1	0	0	0	0	1	0	0	0	1	0	0	?	0	?	?	1	1	0	1	1	1	1	1	2	2	0	0	?	0	0	0	1	0	1	1	1	2	0	?	?	1	0	0	?	1	1	0	0	0	0	1	1
***Siphoneugena guilfoyleiana* Proença**	0	0	0	0	0	1	?	0	0	0	0	?	2	0	0	0	0	0	0	0	?	0	?	0	1	1	0	?	0	0	0	1	1	0	0	0	1	0	0	0	1	0	0	?	?	?	?	1	1	0	1	1	1	1	1	2	2	?	?	?	0	0	1	1	?	1	1	?	2	0	?	?	1	?	?	0	?	?	?	0	0	?	1	1
***Syzygium jambos* (L.) Alston**	0	0	2	0	0	0	2	0	0	0	1	0	2	0	0	1	1	0	0	0	?	0	1	1	0	0	1	1	0	1	0	0	0	0	0	1	1	0	0	1	1	0	1	0	2	1	0	2	1	0	0	0	1	2	0	0	0	1	0	1	?	2	0	0	0	?	1	?	1	?	0	0	0	0	1	1	1	1
***Tristaniopsis laurina* (Sm.) Peter G.Wilson & J.T.Waterh.**	0	0	0	0	0	0	?	0	0	0	1	0	0	0	0	1	1	0	0	0	1	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	1	1	0	1	1	0	0	1	0	0	3	0	0	?	0	?	0	1	0	0	0	0	0	2	2	1	1	1
***Ugni candollei* (Barnéoud) O.Berg**	1	0	0	1	0	0	?	0	1	0	1	0	0	0	0	1	1	1	0	0	1	0	1	1	0	0	0	1	0	0	0	2	0	2	1	1	0	2	0	0	1	?	2	1	1	0	0	0	0	1	0	0	0	0	?	?	0	0	2	0	1	3	1	2	0	0	1	1	1	1	0	1	1	1	2	1	0	1	0
***Ugni molinae* Turcz.**	1	0	0	0	0	0	?	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	2	1	0	0	1	0	0	0	?	2	1	1	2	0	0	2	1	0	0	0	0	?	?	0	0	2	0	1	3	1	2	0	1	0	1	?	1	0	1	1	1	0	0	0	0	1
***Ugni myricoides* (Kunth) O.Berg**	1	0	0	0	0	0	?	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	?	0	?	0	0	?	0	0	0	2	1	0	?	1	0	0	0	?	2	1	1	2	0	0	2	1	0	0	0	0	?	?	?	0	0	2	0	1	3	1	2	0	?	?	?	?	1	0	?	?	?	0	0	?	0	1
***Ugni selkirkii* (Hook. and Arn.) O.Berg**	1	0	0	0	0	0	?	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	1	0	0	0	0	0	1	0	0	?	1	0	?	1	?	2	1	1	0	0	0	1	0	0	0	0	0	?	?	1	0	2	0	1	3	1	2	0	?	1	1	?	1	0	1	1	?	0	1	?	0	0
***Xanthomyrtus compacta* Diels**	1	1	0	0	0	0	?	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	1	1	1	2	1	0	1	0	0	1	1	0	0	1	0	1	?	0	1	0	1	?	0	2	0	?	?	0	?	?	0	1	1	0	2	1	1	2	1

Table 6.5. DNA site variation and parsimony statistics for each molecular region analysed in the phylogenetic analyses. Nuclear: ETS + ITS, Plastid: *matK* + *psbA-trnH*, Molecular: ETS + ITS + *matK* + *psbA-trnH*, Total: ETS + ITS + *matK* + *psbA-trnH* + morphology

	ETS	ITS	*matK*	*psbA-trnH*	Nuclear	Plastid	Molecular	Morphology	Total
Number of characters	538	785	783	656	1323	1439	2762	79	2841
Number of indels scored	2	5	1	2	7	3	10	NA	10
Number of variable characters	317	338	186	328	655	514	1169	79	1248
% of variable characters	59.4	43	23.7	50	49.5	35.7	42.3	100	44
Parsimony-informative characters	229	209	87	121	438	208	646	79	725
% of parsimony-informative characters	42.6	26.6	11.1	18.5	33.1	14.5	23.4	100	25.5
Number of trees retained	>50,000	>50,000	>50,000	>50,000	>50,000	>50,000	>50,000	>50,000	272
Tree length	1,068	1,262	271	438	2,386	727	3192	654	4917
Consistency Index (CI)	0.44	0.41	0.79	0.65	0.41	0.69	0.46	0.19	0.38
Retention Index (RI)	0.62	0.55	0.85	0.79	0.57	0.79	0.61	0.65	0.53

Table 6.6. Statistical support for main clades in this investigation (PP/ML/MP/MR). PP= Bayesian posterior probability ML= Maximum likelihood bootstrap support, MP= Parsimony bootstrap support, MR= % majority rule.

Clade	Without indels PP/ML/MP/MR	With indels PP/ML/MP/MR
Plastid		
Tribe Myrteae monophyletic	0.88/60/99/100	0.90/60/100/100
Myrtus sister to all other Myrteae	0.57/-/-/100	0.56/-/-/100
Myrtus sister to Australasian group	-/-/-/-	-/-/-/-
Amomyrtus monophyletic	-/-/-/-	-/-/-/-
Austromyrtus monophyletic	0.94/80/81/100	1/80/82/100
Blepharocalyx monophyletic	-/-/-/-	-/-/-/-
Eugenia monophyletic	-/-/-/50	-/-/-/50
Lenwebbia monophyletic	1/70/69/100	0.97/70/71/100
Luma monophyletic	0.95/90/94/100	1/91/94/100
Myrceugenia monophyletic	0.61/50/100/100	0.72/50/100/100
Myrceugenia ovata (varieties monophyletic)	-/-/-/-	-/-/-/-
Myrcianthes monophyletic	-/76/77/-	-/76/77/-
Psidium monophyletic	1/50/56/100	1/50/56/100
Siphoneugena monophyletic	1/99/99/100	1/99/100/100
Ugni monophyletic	-/-/-/-	-/-/-/-
Myrcianthes coquimbensis sister to all other *Myrcianthes*	-/-/-/-	-/-/-/-
Myrceugenia rufa sister to all *Myrceugenia*	-/-/-/-	-/-/-/-
Myrcianthes sister to *Eugenia*	-/-/-/-	-/-/-/-
Myrteola sister to *Lophomyrtus+Neomyrtus*	0.81/-/-/100	0.80/-/-/100
Blepharocalyx salicifolius sister to all other SA Myrteae	-/-/-/-	-/-/-/-
Myrceugenia+Luma+Blepharocalyx	-/-/-/-	-/-/-/-
Blepharocalyx+Luma	0.98/-/100/100	1/-/100/100
Blepharcalyx+Nothomyrcia	-/-/-/-	-/-/-/-
Myrceugenia Brazilian clade	-/54/50/50	-/54/50/50
Myrceugenia+Myrcianthes+Eugenia+Amomyrtus+Legrandia	-/-/-/-	-/-/-/-
Ugni candollei+U.selkirkii	1/100/100/100	1/100/100/100
Ugni myricoides+U.molinae	0.95/100/100/100	1/100/100/100
Amomyrtus+Legrandia	-/-/-/-	-/-/-/-
Lophomyrtus+Neomyrtus	0.80/73/74/100	1/73/75/100
Lophomyrtus+Neomyrtus+Lenwebbia	0.85/87/90/100	1/87/90/100
Ugni+Lophomyrtus+Neomyrtus+Lenwebbia+Myrteola	0.85/-/-/100	0.85/-/-/100
Rhodamnia+Austromyrtus+Decasper.+Gossia+Rhodomyrtus	-/-/-/-	-/-/-/-
American Myrteae *+Lophomyrtus+Neomyrtus+Lenwebbia*)	0.87/-/100/100	0.87/-/100/100
"*Myrcia* group" including *Nothomyrcia*	0.90/-/-/-	0.92/-/-/-
Siphoneugena+Plinia+Nothomyrcia	-/-/-/-	-/-/-/-
Psidium+Acca	-/-/-/100	-/-/-/100
Nuclear		
Tribe Myrteae monophyletic	0.99/99/99/100	1/99/99/100

Clade	Without indels PP/ML/MP/MR	With indels PP/ML/MP/MR
Myrtus sister to all other Myrteae	-/-/-/60	-/-/-/60
Myrtus sister to Australasian group	0.91/53/57/100	0.90/55/57/100
Amomyrtus monophyletic	0.98/100/100/100	1/100/100/100
Austromyrtus monophyletic	1/100/100/100	1/100/100/100
Blepharocalyx monophyletic	-/-/-/-	-/-/-/-
Eugenia monophyletic	1/98/99/100	1/98/99/100
Lenwebbia monophyletic	1/100/98/100	1/100/98/100
Luma monophyletic	1/100/100/100	1/100/100/100
Myrceugenia monophyletic	1/98/100/100	1/98/100/100
Myrceugenia ovata (varieties monophyletic)	-/-/-/-	-/-/-/-
Myrcianthes monophyletic	0.95/100/100/100	0.99/100/100/100
Psidium monophyletic	0.98/95/98/100	0.99/97/98/100
Siphoneugena monophyletic	1/100/100/100	1/100/100/100
Ugni monophyletic	-/-/-/-	-/-/-/-
Myrcianthes coquimbensis sister to all other *Myrcianthes*	0.95/97/98/100	1/97/98/100
Myrceugenia rufa sister to all *Myrceugenia*	0.97/67/99/100	0.98/67/99/100
Myrcianthes sister to *Eugenia*	0.91/80/84/100	0.91/80/84/100
Myrteola sister to *Lophomyrtus+Neomyrtus*	-/-/-/-	-/-/-/-
Blepharocalyx salicifolius sister to all other SA Myrteae	-/-/-/-	-/-/-/-
Myrceugenia+Luma+Blepharocalyx	-/-/-/-	-/-/-/-
Blepharocalyx+Luma	-/-/-/-	-/-/-/-
Blepharcalyx+Nothomyrcia	0.67/-/52/50	0.68/-/52/50
Myrceugenia Brazilian clade	0.99/61/63/100	0.99/61/63/100
Myrceugenia+Myrcianthes+Eugenia+Amomyrtus+Legrandia	0.85/51/55/100	0.99/52/55/100
Ugni candollei+U.selkirkii	-/95/100/100	-/95/100/100
Ugni myricoides+U.molinae	-/94/93/100	-/94/93/100
Amomyrtus+Legrandia	1/87/90/100	1/87/90/100
Lophomyrtus+Neomyrtus	1/99/98/100	1/100/98/100
Lophomyrtus+Neomyrtus+Lenwebbia	0.86/99/99/100	0.99/99/99/100
Ugni+Lophomyrtus+Neomyrtus+Lenwebbia+Myrteola	-/-/-/-	-/-/-/-
Rhodamnia+Austromyrtus+Decasper.+Gossia+Rhodomyrtus	-/-/-/100	-/-/-/100
American Myrteae *+Lophomyrtus+Neomyrtus+Lenwebbia*)	-/-/-/-	-/-/-/-
"*Myrcia* group" including *Nothomyrcia*	-/-/-/-	-/-/-/-
Siphoneugena+Plinia+Nothomyrcia	-/-/-/50	-/-/-/50
Psidium+Acca	1/64/70/100	0.98/64/70/100
Molecular combined		
Tribe Myrteae monophyletic	0.91/100/100/100	1/100/100/100
Myrtus sister to all other Myrteae	0.73/61/65/100	0.73/63/65/100
Myrtus sister to Australasian group	-/-/-/-	-/-/-/-
Amomyrtus monophyletic	1/78/83/100	0.96/78/83/100
Austromyrtus monophyletic	0.99/100/100/100	1/100/100/100
Blepharocalyx monophyletic	-/-/-/-	-/-/-/-
Eugenia monophyletic	1/98/90/100	1/98/99/100
Lenwebbia monophyletic	0.98/94/100/100	1/100/100/100

Clade	Without indels PP/ML/MP/MR	With indels PP/ML/MP/MR
Luma monophyletic	1/99/95/100	1/99/98/100
Myrceugenia monophyletic	1/98/100/100	1/98/100/100
Myrceugenia ovata (varieties monophyletic)	-/-/-/-	-/-/-/-
Myrcianthes monophyletic	1/89/87/100	0.99/86/87/100
Psidium monophyletic	0.92/100/100/100	1/100/100/100
Siphoneugena monophyletic	1/99/99/100	1/100/100/100
Ugni monophyletic	-/-/-/-	-/-/-/-
Myrcianthes coquimbensis sister to all other *Myrcianthes*	1/100/100/100	1/100/100/100
Myrceugenia rufa sister to all *Myrceugenia*	0.56/-/99/100	0.53/-/99/100
Myrcianthes sister to *Eugenia*	1/100/100/100	1/100/100/100
Myrteola sister to *Lophomyrtus+Neomyrtus*	0.53/-/-/-	0.56/-/-/-
Blepharocalyx salicifolius sister to all other SA Myrteae	0.70/-/-/-	0.70/-/-/-
Myrceugenia+Luma+Blepharocalyx	0.98/-/-/-	0.98/-/-/-
Blepharocalyx+Luma	0.60/-/-/-	0.60/-/-/-
Blepharcalyx+Nothomyrcia	-/-/72/70	-/-/72/70
Myrceugenia Brazilian clade	1/93/95/100	1/93/95/100
Myrceugenia+Myrcianthes+Eugenia+Amomyrtus+Legrandia	-/-/-/-	-/-/-/-
Ugni candollei+U.selkirkii	0.96/99/100/100	0.99/99/100/100
Ugni myricoides+U.molinae	1/-/-/100	1/-/-/100
Amomyrtus+Legrandia	0.95/70/72/70	0.95/70/72/70
Lophomyrtus+Neomyrtus	0.98/100/100/100	1/100/100/100
Lophomyrtus+Neomyrtus+Lenwebbia	0.95/99/100/100	1/100/100/100
Ugni+Lophomyrtus+Neomyrtus+Lenwebbia+Myrteola	-/-/-/60	-/-/-/60
Rhodamnia+Austromyrtus+Decasper.+Gossia+Rhodomyrtus	0.83/50/53/70	0.83/50/53/70
American Myrteae *+Lophomyrtus+Neomyrtus+Lenwebbia*)	0.95/57/55/100	0.95/57/55/100
"*Myrcia* group" including *Nothomyrcia*	0.74/-/-/-	0.74/-/-/-
Siphoneugena+Plinia+Nothomyrcia	0.95/50/55/70	0.95/50/55/70
Psidium+Acca	0.98/62/69/100	0.99/67/69/100
Morphology		
Tribe Myrteae monophyletic	-/51/53/100	-/51/53/100
Myrtus sister to all other Myrteae	-/- /-/-	-/- /-/-
Myrtus sister to Australasian group	-/-/-/-	-/-/-/-
Amomyrtus monophyletic	-/73/75/100	-/71/75/100
Austromyrtus monophyletic	-/80/84/100	-/80/84/100
Blepharocalyx monophyletic	-/60/100/100	-/60/100/100
Eugenia monophyletic	-/54/57/100	-/54/57/100
Lenwebbia monophyletic	-/92/97/100	-/92/97/100
Luma monophyletic	-/-/-/100	-/-/-/100
Myrceugenia monophyletic	-/50/100/100	-/50/100/100
Myrceugenia ovata (varieties monophyletic)	-/60/60	-/60/60
Myrcianthes monophyletic	-/- /-/-	-/- /-/-
Psidium monophyletic	-/- /-/100	-/- /-/100
Siphoneugena monophyletic	-/-/-/50	-/-/-/100
Ugni monophyletic	-/-/-/-/-	-/-/-/-/-

Clade	Without indels PP/ML/MP/MR	With indels PP/ML/MP/MR
Myrcianthes coquimbensis sister to all other *Myrcianthes*	-/-/-/-	-/-/-/-
Myrceugenia rufa sister to all *Myrceugenia*	-/- /-/-	-/- /-/-
Myrcianthes sister to *Eugenia*	-/- /-/-	-/- /-/-
Myrteola sister to *Lophomyrtus+Neomyrtus*	-/- /-/100	-/- /-/100
Blepharocalyx salicifolius sister to all other SA Myrteae	-/- /-/-	-/- /-/-
Myrceugenia+Luma+Blepharocalyx	-/-/99/100	-/-/99/100
Blepharocalyx+Luma	-/56/99/100	-/56/98/100
Blepharcalyx+Nothomyrcia	-/-/-/-	-/-/-/-
Myrceugenia Brazilian clade	-/- /-/-	-/- /-/-
Myrceugenia+Myrcianthes+Eugenia+Amomyrtus+Legrandia	-/- /-/-	-/- /-/-
Ugni candollei+U.selkirkii	-/-/-/-	-/-/-/-
Ugni myricoides+U.molinae	-/90/99/100	-/97/99/100
Amomyrtus+Legrandia	-/- /-/-	-/- /-/-
Lophomyrtus+Neomyrtus	-/-/-/100	-/-/-/100
Lophomyrtus+Neomyrtus+Lenwebbia	-/- /-/-	-/- /-/-
Ugni+Lophomyrtus+Neomyrtus+Lenwebbia+Myrteola	-/- /-/55	-/- /-/55
Rhodamnia+Austromyrtus+Decasper.+Gossia+Rhodomyrtus	-/-/-/-	-/-/-/-
American Myrteae *+Lophomyrtus+Neomyrtus+Lenwebbia*)	-/-/-/-	-/-/-/-
"*Myrcia* group" including *Nothomyrcia*	-/-/-/-	-/-/-/-
Siphoneugena+Plinia+Nothomyrcia	-/- /-/-	-/- /-/-
Psidium+Acca	-/- /-/-	-/- /-/-
Total evidence		
Tribe Myrteae monophyletic	1/95/99/100	1/98/99/100
Myrtus sister to all other Myrteae	0.76/-/-/-	0.76/-/-/-
Myrtus sister to Australasian group	-/100/98/100	-/100/98/100
Amomyrtus monophyletic	1/100/99/100	1/100/99/100
Austromyrtus monophyletic	0.99/100/99/100	1/100/99/100
Blepharocalyx monophyletic	-/-/-/-	-/-/-/-
Eugenia monophyletic	1/100/99/100	1/100/99/100
Lenwebbia monophyletic	0.95/95/99/100	1/100/99/100
Luma monophyletic	1/100/99/100	1/100/99/100
Myrceugenia monophyletic	1/98/99/100	1/100/99/100
Myrceugenia ovata (varieties monophyletic)	-/-/-/-	-/-/-/-
Myrcianthes monophyletic	0.99/100/99/100	1/100/99/100
Psidium monophyletic	1/100/94/100	1/100/99/100
Siphoneugena monophyletic	0.99/99/95/100	1/100/95/100
Ugni monophyletic	-/-/-/-	-/-/-/-
Myrcianthes coquimbensis sister to all other *Myrcianthes*	1/100/99/100	1/100/99/100
Myrceugenia rufa sister to all *Myrceugenia*	-/-/-/-	-/-/-/-
Myrcianthes sister to *Eugenia*	0.99/73/73/64	1/73/73/64
Myrteola sister to *Lophomyrtus+Neomyrtus*	-/-/-/-	-/-/-/-
Blepharocalyx salicifolius sister to all other SA Myrteae	0.83/81/95/100	0.8/82/99/100
Myrceugenia+Luma+Blepharocalyx	0.90/-/-/-	0.91/-/-/-
Blepharocalyx+Luma	0.91/96/95/100	0.91/92/95/100

Clade	Without indels PP/ML/MP/MR	With indels PP/ML/MP/MR
Blepharcalyx+Nothomyrcia	-/-/-/-	-/-/-/-
Myrceugenia Brazilian clade	1/-/-/-	1/-/-/-
Myrceugenia+Myrcianthes+Eugenia+Amomyrtus+Legrandia	-/-/-/-	-/-/-/-
Ugni candollei+U.selkirkii	1/99/97/50	1/99/98/50
Ugni myricoides+U.molinae	0.95/94/100/50	1/99/100/50
Amomyrtus+Legrandia	0.99/92/94/50	0.99/92/94/50
Lophomyrtus+Neomyrtus	1/-/-/100	1/-/-/100
Lophomyrtus+Neomyrtus+Lenwebbia	1/100/99/100	1/100/99/100
Ugni+Lophomyrtus+Neomyrtus+Lenwebbia+Myrteola	0.97/-/-/60	0.95/-/-/60
Rhodamnia+Austromyrtus+Decasper.+Gossia+Rhodomyrtus	0.81/100/99/100	0.8/100/99/100
American Myrteae *+Lophomyrtus+Neomyrtus+Lenwebbia*)	0.75/51/56/80	0.75/51/56/80
"*Myrcia* group" including *Nothomyrcia*	0.92/53/57/50	0.89/53/57/50
Siphoneugena+Plinia+Nothomyrcia	0.77/-/-/-	0.76/-/-/-
Psidium+Acca	0.99/-/-/60	1/-/-/60

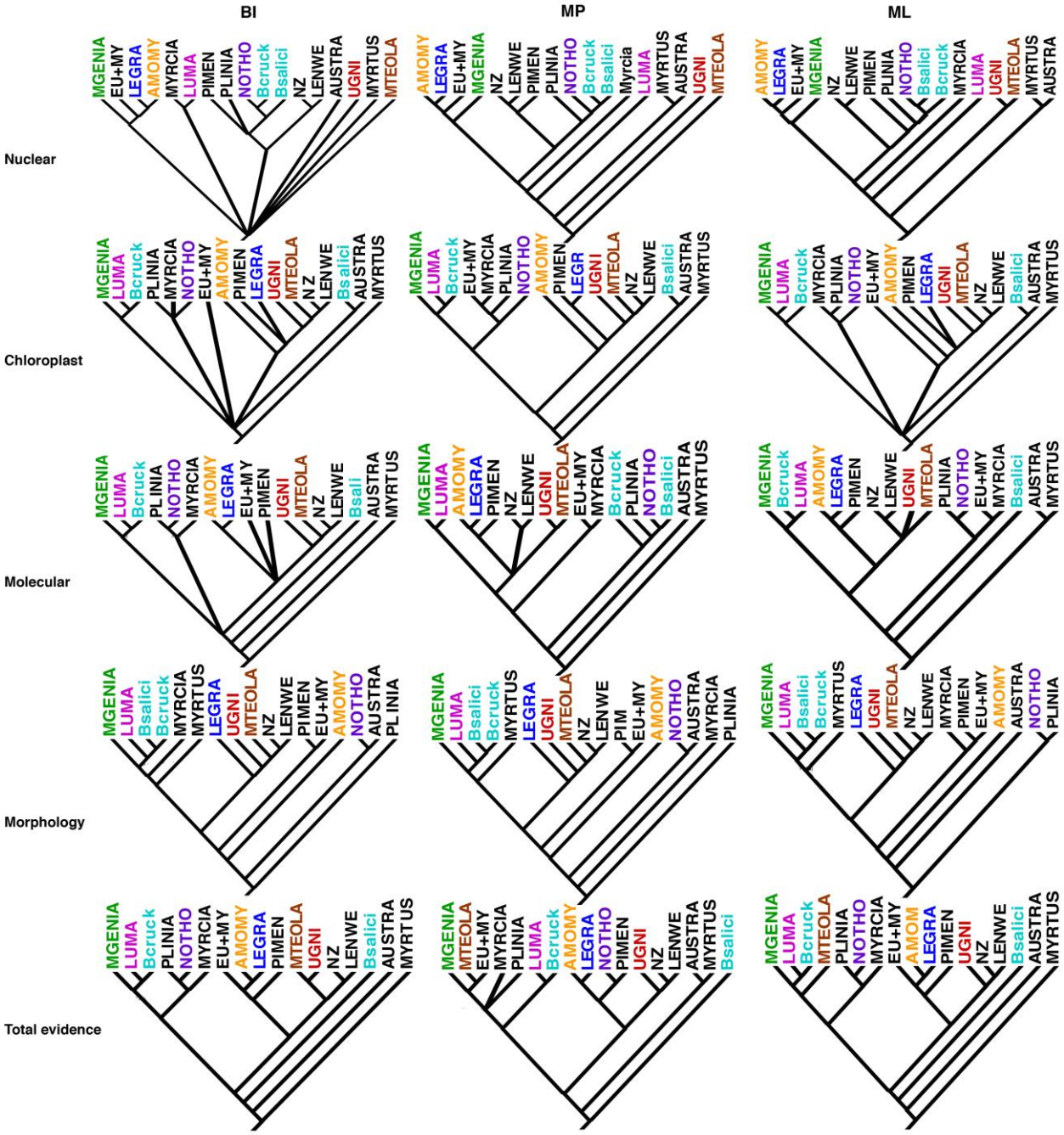


Figure 6.1. Summary of phylogenetic relationships within Myrteae found in Bayesian inference (BI), parsimony (MP) and maximum likelihood (ML) analyses based on individual and combined data sets. Chilean species and those genera containing Chilean species are highlighted in colour. In all trees, the Chilean species *Myrcianthes coquimbensis* is embedded in the clade EU+MY (*Eugenia*+*Myrcianthes*). Data set abbreviations: Nuclear=ETS+ITS, Chloroplast=*psbA-trnH*+*mat*K, Molecular=Nuclear+Chloroplast, Total evidence= Nuclear+Chloroplast+Morphology. Clade abbreviations: AMOMY=*Amomyrtus*, AUSTRA=Australasian group, Bcruck=*Blepharocalyx cruckshanksii*, Bsalici=*Blepharocalyx salicifolius*, EU+MY=*Eugenia*+*Myrcianthes*, LEGRA=*Legrandia*, LENWE=*Lenwebbia*, LUMA=*Luma*, MGENIA=*Myrceugenia*, MTEOLA=*Myrteola*, MYRCIA=*Myrcia* group, MYRTUS=*Myrtus*, NZ=*Lophomyrtus*+*Neomyrtus*, NOTHO=*Nothomyrcia*, PIMEN=*Pimenta* group, PLINIA=*Plinia* group, UGNI=*Ugni*.

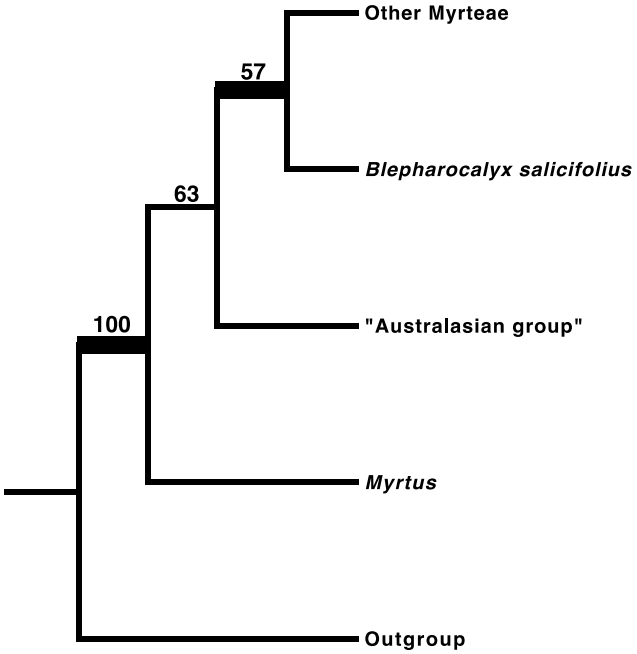


Figure 6.2. General phylogenetic relationships in Myrteae recovered in this study showing the monophyly of the tribe and the sister group relationship between *Myrtus* and the rest of the tribe. Clades having bold branches show Bayesian posterior probabilities greater than 0.95. Numbers above branches indicate ML bootstrap support above 50%.

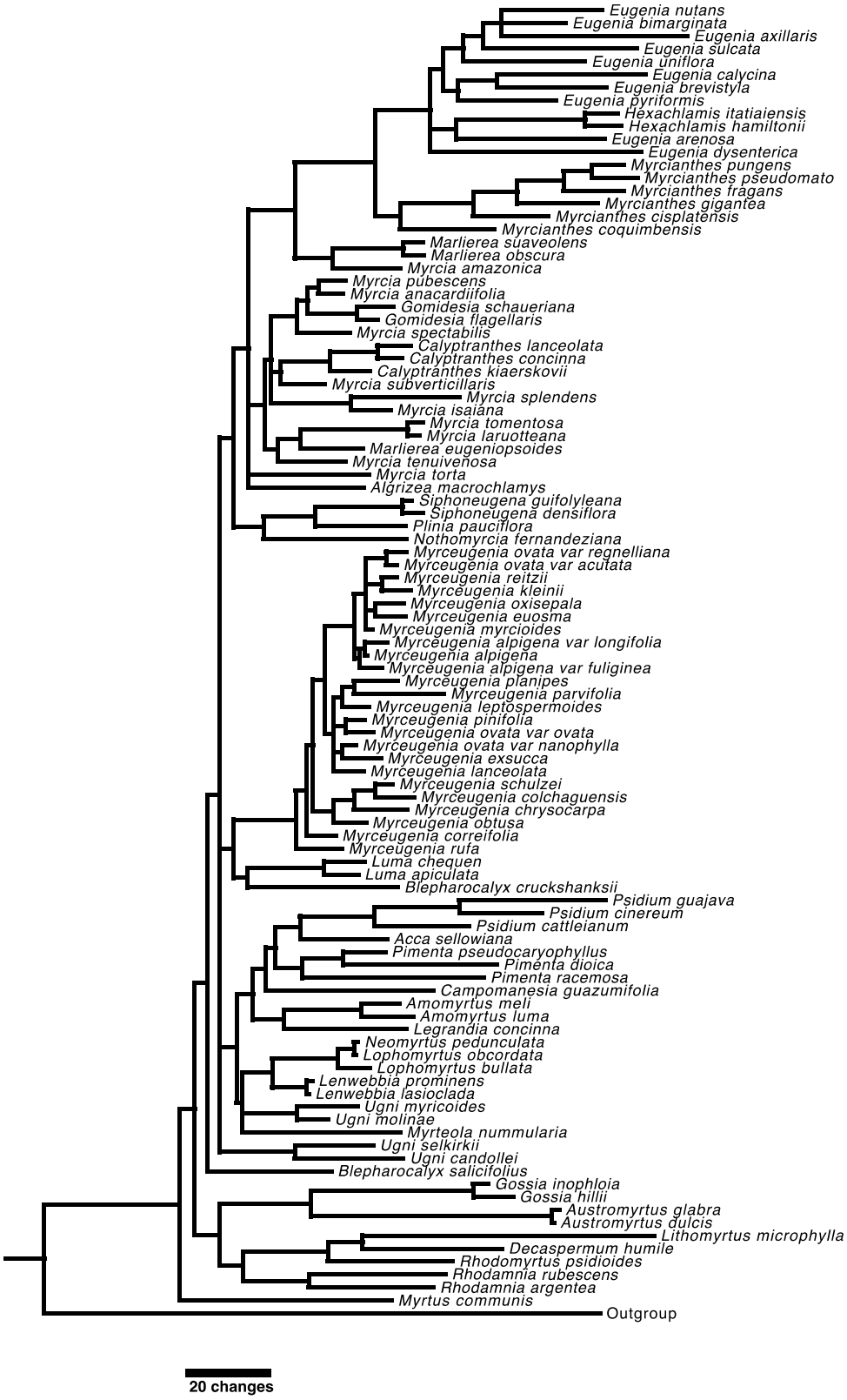


Figure 6.3. Randomly selected phylogram (BI) of the tribe Myrteae based on four combined DNA regions (ETS, ITS, *matK*, *psbA-trnH* spacer).

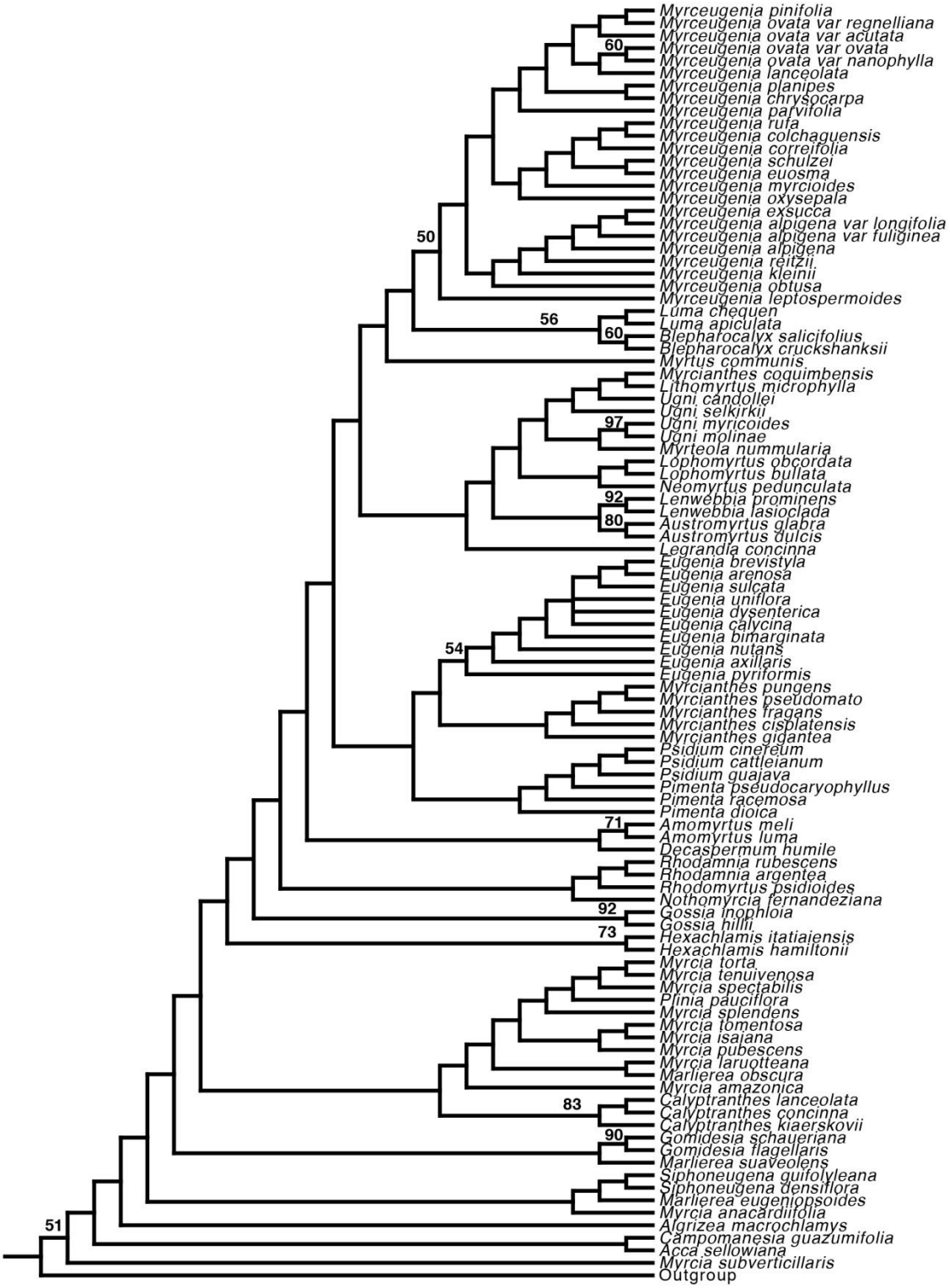


Figure 6.4. Phylogenetic relationships (MP) of the tribe Myrteae based on morphological data. Numbers above branches indicate bootstrap support above 50%.

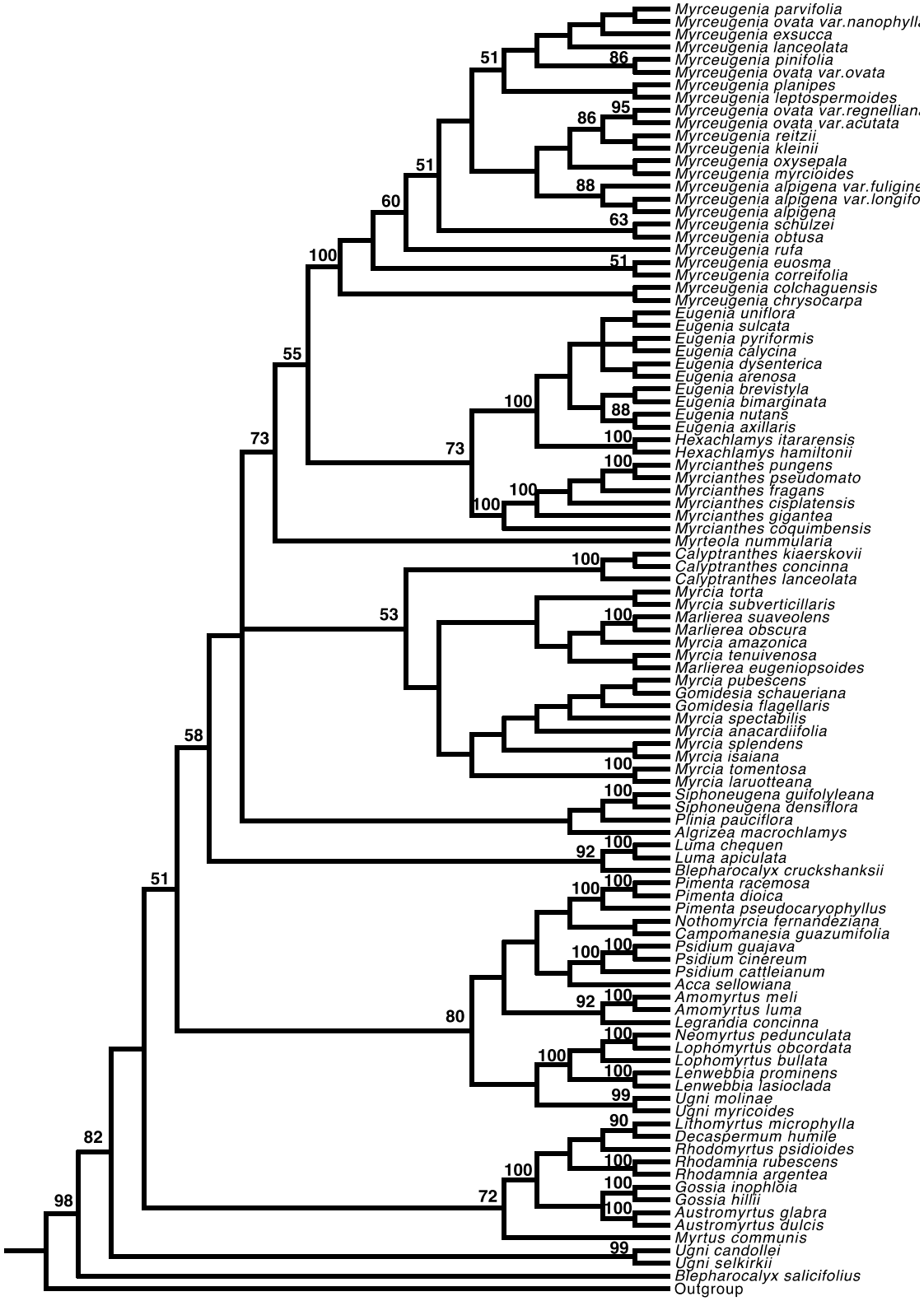


Figure 6.5. Phylogenetic relationships of the tribe Myrteae based on a maximum parsimony analysis of four combined DNA regions (ETS, ITS, *matK*, *psbA-trnH* spacer) and 79 morphological characters. Numbers above branches indicate ML bootstrap support above 50%.

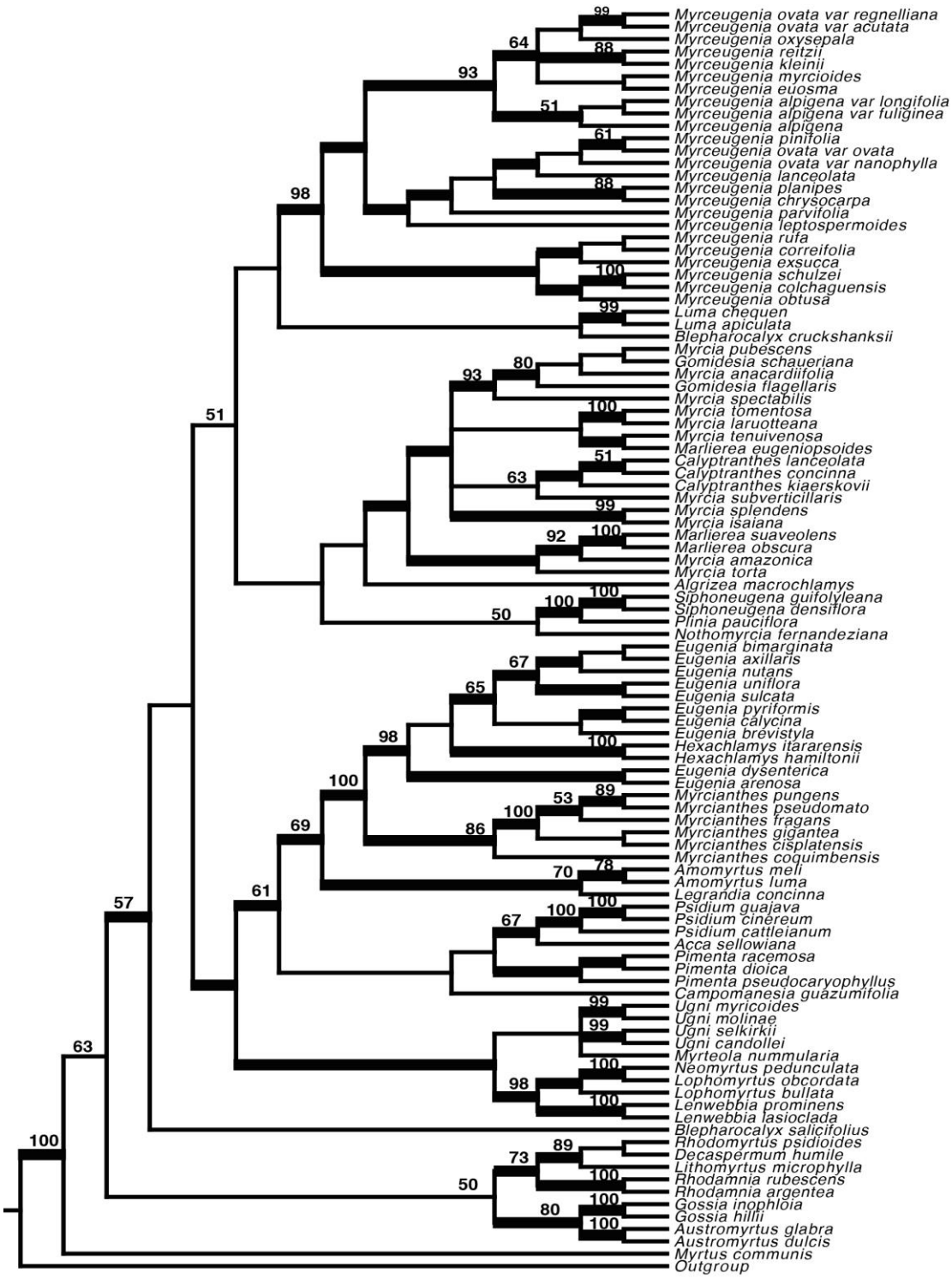
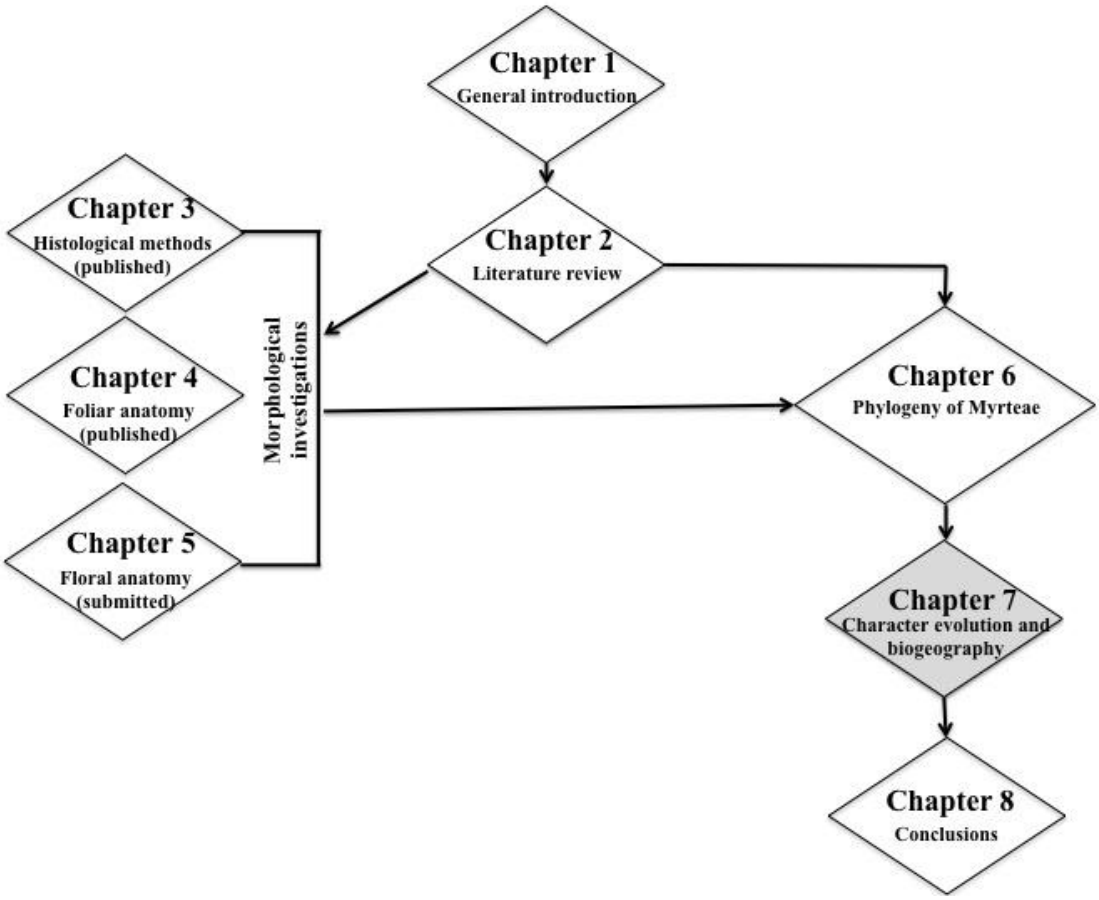


Figure 6.6. Phylogenetic relationships of the tribe Myrteae based on a Bayesian analysis of four combined DNA regions (ETS, ITS, *matK*, *psbA-trnH* spacer) and a set of 79 morphological characters. Numbers above branches indicate ML bootstrap support above 50% and bold braches indicate PP values above 0.95.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CHAPTER 7: Character evolution in Myrteae (Myrtaceae)

Abstract

The tribe Myrteae is the richest tribe in the Myrtaceae in terms of genera and one of the most abundant components of in the forests of South America, eastern Australia and Australasia. Species of Myrteae normally occur in rainforests, humid temperate forests or flooded environments, but some species occur in coastal sands, shrublands and semi-dry habitats. Myrteae displays a diversity of vegetative and reproductive character states but there is a lack of understanding regarding morphological evolution in the tribe. The evolution of 75 morphological characters and ancestral biogeographic areas were studied using reconstruction of ancestral states based on maximum parsimony. Analyses included gross morphological, micromorphological, anatomical and histochemical characters related to leaves, flowers, fruits and secondary xylem. Some notable morphological synapomorphies include dibrachiate hairs in *Myrceugenia*, anisocytic stomata in the *Lophomyrtus+Neomyrtus* clade and the genus *Austromyrtus*, a circular leaf vascular complex in *Amomyrtus*, *Calyptranthes* and *Luma* and abundant tannins in the phloem of flowers in the *Luma+Blepharocalyx* clade. Although many characters were optimized as synapomorphies in some groups, the evolution of several characters is highly homoplasious. The diversification of morphological characters involves character states that may confer ecological capabilities to some species, particularly those occurring in xerophytic habitats (e.g., *Myrceugenia rufa*, *Myrcianthes coquimbensis*). A number of character states potentially related to evolution in xerophytic habitats such as presence of hypodermis, multiple epidermis and straight epidermal anticlinal walls might be products of convergent evolution. Ancestral state reconstruction of biogeographic areas supports the hypothesis of a Gondwanan origin for Myrteae in Australasia with migration to South America and several dispersal events. The lineages could have spread from southern South America into other regions of the continent. The restricted presence of the endemics, *Myrcianthes coquimbensis* in the coast of north-central Chile and *Ugni candollei* in the coast of southern Chile, might be result of dispersal events or remnants of a past extended distribution of the genera.

7.1 Introduction

The tribe Myrteae (Myrtaceae) comprises approximately 2400 species in 54 genera and encompasses only fleshy-fruited species (Lucas et al., 2007). Myrteae has a postulated Gondwanan origin (Sytsma et al., 2004; Lucas et al., 2007) and is mainly distributed in the southern hemisphere, with the majority of species occurring in South America, Central America and Australasia (Wilson, 2011). Other areas with fewer species of Myrteae are Africa, China, India, Mexico, Florida and the Mediterranean (Wilson, 2011). The majority of Myrteae species occur in rainforests and humid habitats of South America and Australia, however some species occur in drier habitats, such as coastal sands, bushlands and shrublands (Wilson, 2011). A few species, such as *Myrceugenia rufa* and *Myrcianthes coquimbensis* occur exclusively in drier habitats with the water supply limited to fog and ocean breeze (Landrum and Grifo, 1988; Wilson, 2011). Some Myrteae, including those occurring in drier habitats, have been considered xerophytic based on gross morphological characters (Cardoso et al., 2009; Wilson, 2011).

Along with gross morphology, morpho-anatomical characters have been considered taxonomically informative in Myrtaceae. Metcalfe & Chalk (1979), Schmid (1980) and Keating (1984) described leaf anatomical characters at family level and emphasized the taxonomic implications of these features. Anatomical characters such as midrib shape, adaxial and abaxial phloem confluence and presence of hypodermis have been reported as useful to delimitate species of South American Myrteae, alongside gross morphological characters (Cardoso et al., 2009). Combination of stomatal complexes, type of crystals and adaxial phloem partition have been considered taxonomically informative for the Australasian genus *Syzygium*, allowing reliable identification of subgeneric groups (Soh & Parnell, 2011). Leaf micromorphological characters, including stomatal complexes and anticlinal walls of epidermal cells, have been used to diagnose *Eugenia* species occurring in mesic and drier habitats (Fontenelle et al., 1994; Haaron and Moore, 1996). Anatomical and micromorphological investigations have contributed to characterize species of Myrteae occurring in dry habitats (Cardoso et al., 2009; Gomes et al., 2009; Retamales et al., 2015; Retamales and Scharaschkin, 2015). However, anatomical and micromorphological characters described in previous investigations have never been assessed in a phylogenetic context.

Some species of Myrteae possess character states considered plesiomorphic for the family. Some of these features are scalariform perforation plates in some species of *Myrteola*, *Myrceugenia*, *Luma*, *Ugni* and *Tepualia*, helical wall thickenings in *Myrceugenia* (Schmid and Baas, 1984; Landrum, 1981b), tracheids in *Luma*, *Blepharocalyx* and *Amomyrtus* (Ragonese, 1976), and small embryos that allow more storage of nutrients in the seed of *Ugni* (Landrum and Stevenson, 1986). Scalariform perforated plates in vessels have been attributed to primitive species (Stern, 1978), being supposedly an adaptation to cooler or mountain environments (Jansen et al., 2004). Scalariform perforation plates on wood vessel elements are more common in montane and submontane Andean species of Myrteae (Lucas et al., 2007). The shared presence of scalariform perforation plates in South American Myrtaceae (*Myrceugenia*, *Myrteola*, *Metrosideros*) and *Neomyrtus* and *Lophomyrtus* from New Zealand has been used to suggest taxonomic relationships between these genera (Lucas et al., 2007). Simple hairs are considered a plesiomorphic character state for the family, with other types of unicellular hairs and multicellular hairs having evolving later (Wilson et al., 2001).

Character evolution studies are scarce in Myrteae, with only a limited number of morphological and anatomical characters investigated. Lucas et al. (2007) traced the evolution of four morpho-anatomical characters in Myrteae, namely placentation, embryo type, number of ovules and type of vessel perforations. The previous four characters have been indicated as homoplasious, but combinations of these characters have been recognized as useful for clade diagnosis in Myrteae (Lucas et al., 2007). Evolution of pollen characters has been comprehensively assessed for Myrtaceae and the tribe Myrteae and a number of characters have been recognised as highly homoplasious (e.g., pollen width, exine pattern morphology) (Thornhill et al., 2012b, 2012c, 2012d, 2012e). A few gynoecium characters (e.g., number of receptacular bundles, placenta vasculature) have been indicated as potentially informative in the evolution of Myrteae (Pimentel et al., 2014). Stamen shape has been indicated as a phylogenetically informative character, since erect stamens are present in most clades of the tribe, while semi-curved and strongly curved patterns are potential synapomorphies for some clades (Vasconcelos et al., 2015).

Myrteae exhibits a diversity of vegetative and reproductive features, some of them associated with species adapted to dry environments and others considered plesiomorphic in Myrtaceae, but there is a lack of understanding regarding morphological evolution in the tribe. A comprehensive character evolution study based on different types of characters (e.g., reproductive, vegetative) has not been conducted in Myrteae. This chapter studied the

evolution of most of the morphological characters available in the literature plus personal observations described in Chapters 4 and 5 of this thesis. Reconstruction of biogeographic areas was added as another multistate character in the analysis.

7.2 Material and methods

7.2.1 Material examined

Eighty-three species representing 40 genera of Myrtaceae, of which 73 species and 32 genera belong to Myrteae, were investigated. Some species used in the phylogenetic reconstruction of this investigation (refer to Chapter 6) were removed for this analysis for having a high ratio of missing data across the characters. Unlike phylogenetic reconstruction, ancestral state reconstruction is more sensitive to missing data and results might be misleading if species without enough data are included (Wiens, 2000; Prevosti and Chemisquy, 2010). All 111 species analysed in Chapter 6 of this thesis were used for the reconstruction of biogeographic areas, since information for all species is available. Taxonomic sampling was designed to represent all the major clades identified in previous phylogenetic studies in Myrteae (Lucas et al., 2007; Murillo et al., 2013, 2016) and intertribal studies for the outgroup (Biffin et al., 2010; Thornhill et al., 2015). Samples for morphological descriptions were either collected fresh or obtained from herbarium specimens (BRI, NSW, CONC). Vouchers of Chilean Myrteae collected in this study are currently deposited in the Faculty of Forest Sciences Herbarium, University of Chile (EIF), with duplicates housed in the Queensland Herbarium, Brisbane, Australia (BRI). Specimen and voucher information is detailed in the appendices (Appendix 1).

7.2.2 Character selection and justification

Definition of characters, modifications from literature characters and sources of information are presented in Appendix 2. Morphological characters of Myrteae examined in earlier studies formed the basis for the starting point of constructing the morphological data set ((Schmid (1972), Johnson and Briggs (1984), Landrum (1981, 1988a, 1988b), Wilson et al. (2001), Lucas et al. (2007), Cardoso et al. (2009), Soh and Parnell (2011), Pimentel et al. (2014), Vasconcelos et al. (2015)). Characters investigated by Metcalfe and Chalk (1979), Keating (1984) and Wilson (2001, 2011) were added as potentially informative for the outgroups. Those characters that showed variation between outgroup species where included in the data matrix regardless of their variation in Myrteae. Additional characters were added

based on personal observations and publications (Retamales et al., 2014a, 2014b; Retamales and Scharaschkin, 2015). Most of the literature characters were used directly without modifications in character states, while others where modified and adapted to the current taxon sampling. Character states for many characters were generated from personal observations for Chilean and other Myrteae (Retamales et al., 2014a, 2014b; Retamales and Scharaschkin, 2015), but some character states were also gathered from literature. Micromorphological and anatomical characters for non-Chilean Myrteae will be described in this chapter, since they were not described in previous chapters of the thesis. The inclusion of correlated characters that would lead to overweighting of dependent features was avoided in this investigation. Delimitation of character states was conducted following the guidelines of Wiens (2000) and Stevens (1991).

In all, 75 morphological characters were used in this study. Fifty-four characters were taken from other studies; seven of which were modified and 14 were described for the first time and scored. Four characters used in the phylogenetic analyses of this thesis (Chapter 6) with a high ratio of missing data across species were excluded from character evolution analyses, namely type of foliar colleters, number of carpels, silica in wood rays and vessel aggregation. From the character matrix, 47 are binary characters and 28 are unordered multistate; 12.1% are scored as missing data.

7.2.3 Micromorphological and anatomical methods

For micromorphological (SEM) descriptions, leaf and flower material fixed in FAA was dehydrated using a graded ethanol series and then critical point dried (Anderson, 1951) in an Autosamdri-815 automatic critical point drier (Tousimis, Rockville, USA). Samples were mounted on stubs with self-adhesive double-sided carbon discs and sputter-coated with gold palladium for 175 sec using a Leica EM SCD005 Gold Coater (Leica Microsystems, Macquarie Park, NSW, Australia). Examination and documentation of images was carried out using a FEI Quanta 200 SEM/ESEM (FEI, Hillsboro, Oregon, USA) operated at 10kV.

For anatomical descriptions, FAA-fixed material was dehydrated through a graded ethanol series and embedded in paraffin wax (Johansen, 1940; Ruzin, 1999). Transverse sections of leaves were cut using a Leica RM2245 rotary microtome (Leica Microsystems, Macquarie Park, NSW, Australia) at 5µm. Staining of sections was performed using the stains Ruthenium red (0.05% aqueous solution), Toluidine blue (TBO) (0.1% aqueous solution), Safranin O (1% alcoholic solution) and Alcian blue, alone or combined according to standard

staining protocols (Ruzin, 1999; Retamales and Scharaschkin, 2014). In order to reliably identify the chemical compounds in tissues, additional histochemical tests were performed in unstained leaves using the reagents Sudan IV, Chlorazol black E and Phloroglucinol (20% HCl) to detect lipophilic substances and lignin. The chemical nature of leaf intracellular crystals was tested by adding 1µl of acetic acid and 1µl of hydrochloric acid to sections (Maclean and Ivimey-Cook, 1952). Sections were mounted using DPX (Sigma-Aldrich Co., St. Louis, Missouri, USA).

Leaf clearings were prepared by immersing 1-2 cm^2 pieces of leaf material in 10% KOH at room temperature for 48 h followed by 7% NaClO for 2 h or until leaves turned transparent (Gardner, 1975). Cleared leaves were washed five times with distilled water, stained with 1% safranin O and mounted with Lactoglycerol (lactic acid-glycerol 1:1). Slides were observed using a Nikon eclipse 50i compound microscope and images captured using the Nikon NIS-Elements imaging software (Nikon Instruments Inc., Amsterdam, Netherlands).

7.2.4 Ancestral state reconstruction

The phylogenetic tree used as analytical framework for character evolution was the Bayesian majority rule tree based on the total evidence data set of chapter 6 (Fig. 6.6), chosen on basis of resolution and statistical support. The rooting of the tree was based on the constraint outgroup relationships used in the phylogenetic analyses.

Ancestral state reconstruction was conducted using Mesquite v3.10 (Maddison and Maddison, 2016). Parsimony optimizations for all 75 characters were conducted on the selected tree with the option Trace character history of Mesquite v3.10. Those species without information were shown as missing data (hatched lines on branches) instead of being interpolated. Character interpolation might lead to ambiguity of the results, since missing data branches do not interfere with the reconstruction of adjacent character states (Wiens, 2000). The reasoning of optimizing characters on phylogenetic trees constructed in part by morphological characters has been criticised over the years arguing a circular logic (e.g., Hedges and Maxson, 1996). However, the character optimization process optimizes characters one at a time on the phylogenetic tree (Maddison and Maddison, 2016) that was reconstructed using several morphological characters and not only the characters being mapped (de Queiroz, 2000, Scharaschkin and Doyle, 2006). Historical biogeography was reconstructed by mapping the current distribution of the species onto the phylogeny. Biogeographic regions were adapted from Good (1974) and Huggett (2004). The Antarctic

region (Good, 1974; Hugget, 2004) includes Patagonia (Chile and Argentina), New Zealand and Southern Temperate Oceanic Islands (e.g., Juan Fernandez Archipelago). In this study and adapted to the taxon sampling, Patagonia and the Juan Fernandez Archipelago were considered as part of Southern South America. Australasia was considered as Australia, south-east Asia and the Pacific Islands. New Zealand was considered as a different region. In all, geographic distributions were atomized into the following biogeographic areas treated as unordered multistate characters: Southern South America (SSA), other regions of South America (SA), Juan Fernandez Archipelago (JF), Australasia (AUS), New Zealand (NZ), North and Central America (NCA) and Mediterranean-North Africa (MNA). Ancestral state reconstruction of biogeographic areas was conducted under the parsimony criterion.

7.3 Results and discussion

In this section, we present the results of the character evolution analysis and discuss implications in the context of previous studies on Myrteae. Even though most of the information regarding definition of characters and character states is presented in Appendix 2, justifications and decisions to practical problems during the process are discussed in this section. Details about characters of non-Chilean Myrteae (not described in previous chapters of the thesis) will be treated here. When possible, characters have been grouped into sets of related characters.

7.3.1 Habit

Habit (character 1) information was obtained from taxonomic revisions and species descriptions for all species in this investigation (e.g., Landrum, 1981b, 1988a, 1988b, Landrum and Kawazaki, 1997; Wilson, 2011). Species of Myrteae are mostly trees or shrubs, but some species have creeping growth habits (Wilson, 2011). Subjective observations in the literature regarding different types of shrubs (e.g., round shrub, low-ramified shrub) were considered as shrub and scored as 1. In the case of trees, only two types of trees were found in the literature (tree and small tree), therefore considered as tree and scored as 0. Only one species in this taxon sampling (*Myrteola nummularia*) has a creeping habit and was scored with the state 2. The ancestral character reconstruction showed that tree is the ancestral state of the tribe Myrteae. The habit shrub has evolved multiple times in most of the clades during the evolution of the tribe.

7.3.2 Leaf morphology

Characters regarding gross morphology of leaves where gathered from taxonomic revisions and species descriptions for all species (e.g., Landrum, 1981b, 1988a, 1988b, Landrum and Kawazaki, 1997; Wilson, 2011). Leaf gross morphological characters in this study are related to phyllotaxy, pubescence and venation. The phylogenetic and taxonomic significance of the leaf gross characters used in this study was highlighted by Johnson and Briggs (1984) and Wilson et al. (2001). Other leaf gross morphological characters such as type of leaf base, leaf apex, leaf shape, leaf length and type of margin where not included for being extremely variable at intraspecific level in several Myrtaceae (Landrum, 1981a, Johnson and Briggs, 1984; Soh and Parnell, 2011). Vegetative phyllotaxy (character 3) has three character states, namely opposite, alternate and verticillate. A number of species in Myrtaceae are described as having sub-opposite leaves, which was considered as opposite in this study. All Myrteae have opposite (or sub-opposite) leaves, while some species in the outgroup have either opposite, alternate or verticillate leaves. Abaxial leaf pubescence (character 4) was scored based on taxonomic revisions and personal observation of SEM samples generated in this investigation. The ancestral state of abaxial leaf pubescence in *Myrceugenia* is equivocal, since might be sparse-lacking or dense. A dense layer of hairs on the abaxial surface has evolved independently at least five times during the evolution of Myrteae. Leaf pubescence has been considered an important character for the taxonomy and phylogeny of many genera of Myrtaceae (Johnson and Briggs, 1984; Cardoso et al., 2009; Soh and Parnell, 2011). Venation characters have been poorly studied in a phylogenetic context, since scoring of these characters might be difficult and misleading (Wilson et al., 2001). In this investigation, we selected only two venation-related characters mainly due to simplicity in scoring, namely type of primary venation (character 5) and conspicuous/inconspicuous secondary veins (character 6). Types of secondary and tertiary venation are extremely difficult to score accurately in Myrtaceae, mainly due to intraspecific variation (Briggs and Johnson, 1979; Johnson and Briggs, 1984). Ancestral state reconstruction for venation characters showed that basal primary venation is a synapomorphy for *Rhodamnia*, which is an independent evolutionary event in the Myrteae tree. All other species of Myrteae have the typical pinnate venation in Myrtaceae.

7.3.3 Vegetative micromorphology and anatomy

Micromorphological observations (SEM) allowed the identification of three main types of hairs in Myrteae (simple, dibrachiate and glandular) (Fig. 7.1). Simple hairs (character 8) are the ancestral state for Myrteae and Myrtaceae (Wilson et al., 2001), while the absence of simple hairs was only observed in some species of *Myrceugenia*. The presence of simple hairs is the ancestral state in *Myrceugenia.* The presence of dibrachiate hairs was observed in *Myrceugenia, Calyptranthes, Campomanesia* and some species of *Myrcianthes*, *Eugenia* and *Marlierea* (Landrum, 1981b, 1988, Landrum and Kawazaki, 1997; Wilson, 2011). Dibrachiate hairs have appeared independently in Myrteae at least seven times and are a potential synapomorphy for the genus *Myrceugenia* (Fig. 7.1). Glandular hairs on leaves (character 10) have evolved four independent times in Myrteae. Cuticular ornamentation has been studied intensively in some genera of Myrteae, particularly *Eugenia* (Fontenelle et al. 1994, Haron and Moore, 1996). A smooth cuticular surface is the ancestral state for the tribe Myrteae, while the striate cuticular pattern (ornamented cuticle) has arisen at least seven times in the tribe in independent clades. Ancestral state reconstruction of type of stomatal complexes (character 13) showed that the ancestral state for the tribe Myrteae is anomocytic stomata, which is the most common state within the tribe (Fig. 7.2). Anisocytic stomata occur in the Australasian and New Zealand genera of Myrteae, group where is a potential synapomorphy. Paracytic stomata occur in some genera such as *Eugenia, Marlierea, Myrcia, Siphoneugena, Psidium* (Gomes et al., 2009; Cardoso et al., 2009) and the two species of *Luma.* The Chilean species *Ugni candollei* possesses anomocytic and anisocytic stomata even in the same plant (Retamales and Scharaschkin, 2015). Cyclo-staurocytic stomata were observed only in the outgroup *Acmena* (syn: *Syzygium*) *smithii* (Syzygieae). Laterocytic stomata were only observed in *Myrcianthes coquimbensis*, along with paracytic stomata. The interpretation of stomatal types follows van Wyk et al. (1982), Fontenelle et al. (1994), Haron and Moore (1996) and Soh and Parnell (2011) and it is explained in Retamales and Scharaschkin (2015) in detail.

Hypodermis has had at least seven independent origins in Myrteae (Fig. 7.3). Hypodermis was only found in the tribe Myrteae, while multiple epidermis also occurs in *Metrosideros perforata* (Metrosidereae). The parsimony reconstruction shows that a single epidermis with no presence of hypodermis is the ancestral state for Myrteae and the other seven tribes included in this investigation. The presence of multiple epidermis was only observed in *Myrceugenia rufa* and the outgroup species *Metrosideros perforata* (Metrosidereae), which

suggests that this character is an apomorphy of both taxa. The presence of an adaxial hypodermis (character 18) or multiple epidermis (character 19) has been poorly studied in Myrtaceae/Myrteae, with few studies reporting these characters in some species (Cardoso et al., 2009; Soh and Parnell, 2011). Hypodermis is interpreted in this investigation as a layer of large cells located below the epidermis and originated from the ground meristem, while multiple epidermis is generally aligned with the epidermis and originated from ground meristem (Sharma and Mehra, 1972; Dickison, 2000; Martins et al., 2012). Hypodermis and multiple epidermis are considered two independent and non-homologous characters (Martins et al., 2012). One or more layers of hypodermis are generally related to species occurring in environments with high solar radiation (Dickison, 2000; Esau, 1953; Metcalfe and Chalk, 1979).

The number of parenchyma palisade layers (character 23) was scored with three states, namely one layer, two or three layers and four or five layers, based on the ranges of Gomes et al. (2009) and Soh and Parnell (2011). The most common state in Myrteae is two or three layers of palisade parenchyma (Fig. 7.4). The potential ancestral state for the tribe Myrteae is two or three layers. The character state four or five layers of palisade parenchyma has evolved at least three times independently. One layer or palisade parenchyma is a synapomorphy for the Australasian genus *Gossia* and *Eucalyptus*.

Druses (character 24) and prismatic calcium oxalate crystals (character 25) are two important features for Myrteae, since they are present in the majority of species (Cardoso et al., 2009; Wilson, 2011). The presence of rhombohedral crystals is a synapomorphy for the clade formed by New Zealand species + *Lenwebbia* and for the South American genera *Psidium* and *Siphoneugena* (Fig. 7.5). The presence of marginal sclerenchyma (character 26) in leaves of Myrtaceae has been extremely poorly documented. Soh and Parnell (2011) documented a number of species of *Syzygium* with presence of marginal sclerenchyma in some leaves. In this investigation, a number of species of *Myrcianthes*, *Myrcia torta* and some species of the Australasian clade (Australian + Asian species) possess clear and thick strands of sclerenchyma on the margins of leaves (Fig. 7.6). Marginal sclerenchyma apparently does not correspond to veins running across the margins of leaves, since it is mainly composed of fibres without vascular tissue (Evert and Eichhorn, 2006). Large strands of fibres within leaf tissues might be a source of mechanical support for some leaves, particularly coriaceous leaves (Metcalfe and Chalk, 1979). Secretory cavities (character 29) are generally identified for a space surrounded by a sheath of epithelial cells that produce chemical compounds

(Evert and Eichhorn, 2006). Schizogenous secretory cavities are originated by separation of cells and possess fully developed epithelial cells at maturity (Ciccarelli et al., 2008). Lysigenous secretory cavities arise by dissolution of cells and their appearance is a large space without epithelial cells when mature (Esau, 1953). Schizolysigenous cavities occur when cavities are originated for separation of cells (schizogenous origin), but epithelial cells are dissolved at maturity by autolysis (Fahn, 1979; Ciccarelli et al., 2008). Secretory cavities located close to the leaf surface are generally originated from the protodermis, while cavities distributed throughout the mesophyll are originated from the ground meristem (Arruda and Fontenelle, 1994). Secretory cavities are one of the most distinctive characters in Myrtaceae (Wilson et al., 2011), and are often referred as oil dots in field guides and keys. Schizogenous cavities are the most common type and appear as the ancestral state in the tribe Myrteae. Schizolysigenous cavities occur in the New Zealand + *Myrteola nummularia* clade, supporting this phylogenetic relationship.

Several characters related to leaf vascular system were studied in this investigation, namely vascular system shape (character 32), lateral vascular system sheath extension (character 33), adaxial phloem partition (character 34), adaxial-abaxial phloem confluence (character 35) and leaf adaxial phloem abundance (character 36). Ancestral state reconstruction of vascular characters indicated that an ellipsoid main vascular system in leaves is the ancestral state of the tribe Myrteae (Fig. 7.7), and it is a synapomorphy for *Siphoneugena*, *Marlierea*, *Pimenta*, *Rhodamnia*, *Austromyrtus* and *Gossia*. A circular vascular system is a synapomorphy for *Calyptranthes*, *Luma* and *Amomyrtus*, while an arc-shaped vascular complex is a synapomorphy for *Myrceugenia*. A week adaxial phloem partition is the ancestral state in Myrteae. A strong adaxial phloem partition was mainly observed in *Myrceugenia* and species of *Ugni*, except for *Ugni selkirkii*, which has a weak partition. A confluent adaxial and abaxial phloem was found to be the ancestral state in the tribe and a synapomorphy for the New Zealand clade (*Lophomyrtus* + *Neomyrtus* + the Australian *Lenwebbia*) and the genus *Myrceugenia* (Fig. 7.7). Leaf vascular system characters have been cited as taxonomically informative for a number of clades (Schmid, 1984; Gomes et al., 2009; Cardoso et al., 2009; Soh and Parnell, 2011).

Ancestral state reconstruction of wood characters such as presence of perforation plates (character 37), presence of helical wall thickenings (character 39) and presence of fibre-tracheids (character 40) largely reflects what is known for Myrtaceae. Scalariform and mixed simple and scalariform perforation plates were observed in *Myrceugenia, Luma, Ugni* and the

New Zealand *Neomyrtus* and *Lophomyrtus* (Schmid, 1980, Lucas et al., 2007). The "*Myrceugenia* group" (*Myrceugenia*, *Luma*, *Blepharocalyx*) has mixed scalariform-simple perforation plates as ancestral state. The ancestral state of character 37 in the "*Myrteola* group" is equivocal. Helical wall thickenings were mainly observed in *Myrceugenia* and *Myrtus communis*, while the ancestral state for the tribe Myrteae is the presence of simple and uniform thickenings (absence of helical thickenings).

7.3.4 Reproductive morphology, micromorphology and anatomy

Reproductive characters have been regarded as the most significant features for the taxonomy and systematics of Myrtaceae/Myrteae (Schmid, 1972; Cronquist, 1981; Belsham and Orlovich, 2002, 2003; Pimentel et al., 2014; Vasconcelos et al., 2015). Perianth-merosity (number of sepals and petals) has been widely used to discriminate groups of Myrtaceae and support-reject taxonomic affinities (Johnson and Briggs, 1984; Landrum and Grifo, 1988; Wilson, 2011). The number of sepals and petals (character 48) is mostly equivalent in species of Myrtaceae (i.e., species have same number of sepals and petals), except for some species that possess a variable number of perianth segments in the same plant (e.g., *Ugni molinae*). In this investigation, such species were scored with the most common number of sepals and petals observed in vouchers and literature. Pentamerous flowers (5 sepals and 5 petals) is the ancestral state in Myrtaceae and the tribe Myrteae. There is a shift to tetramerous flowers within Myrteae, with a number of reversals back to pentamerous flowers within the tribe (*Amomyrtus*, *Psdium*, *Austromyrtus, Gossia* and *Ugni*) (Fig. 7.8). There are also a number of shifts back from tetramerous to pentamerous flowers within the tribe (Fig. 7.8). Type of embryo is generally homoplasious and some states have evolved multiple times during the evolutionary history of the tribe. A linear or C-shaped hypocotyl with inconspicuous cotyledons was reconstructed as the ancestral state in the tribe Myrteae, with equivocal reconstruction for the South American species. The evolution of type of embryo largely agrees with Lucas et al. (2007) and does not support the previous division of the tribe based on these characters (Snow et al., 2003). The presence of secretory cavities in anthers (anther glands) (character 68) (Fig. 7.9) reflects retention of the ancestral (plesiomorphic) character state in some groups, such as the Australasian clade (Australian + Asian species), the New Zealand clade (*Lophomyrtus* + *Neomyrtus* + *Lenwebbia*) and the *Luma*+*Blepharocalyx* clade. The absence of secretory cavities in anthers is apomorphic for a number of clades within Myrteae, but this character is highly homoplasious.

7.3.5 Histochemistry

Histochemical characters have been poorly documented in Myrteae, since interpretation and identification of chemical compounds is complicated in families with abundant secondary metabolites (Metcalfe and Chalk, 1979; Cronquist, 1981; Keating, 1984). An optimized staining protocol developed in Chapter 3 of this investigation (Retamales and Scharaschkin, 2014) enabled the identification of some compounds that defined a number of histochemical characters. Histochemical content (tannins, other polyphenols, mucilage) was scored as abundant, scarce and absent. Histochemical content was considered abundant when chemical compounds were present into the cells, between cell walls and also in the intercellular spaces. The character was scored as scarce when histochemical content was only present within intercellular spaces. In the case of abundance of polyphenols in the cuticle, the scoring was based on the strength of the staining (i.e., cuticle stained with strong blue suggested abundant polyphenols).

Polyphenols in foliar cuticle (character 76) are extremely abundant in some clades of Myrteae (e.g., New Zealand species + *Ugni*, *Pimenta* spp., *Myrceugenia* spp.), while scarce in most of the species in the tribe and outgroup (Fig. 7.10). Scarce polyphenols in the foliar cuticle are reconstructed as the ancestral state of the tribe. Abundant polyphenols in the cuticle are identified as a strong blue colour (O'Brien et al., 1964) (Fig. 7.10). Mucilage is known to occur in several Myrtaceae (Metcalfe and Chalk, 1979; APG IV, 2016) and is stained red with Ruthenium red (Perez-de-Luque et al., 2006). Mucilage is mainly present in mesophyll and epidermis of the species (Ruzin, 1999; Evert and Eichhorn, 2006) Mucilage content in epidermis (character 78) was defined as scarce, abundant and absent. Scarce mucilage in the epidermis was reconstructed as the ancestral state of the tribe Myrteae and abundant epidermal mucilage has evolved at least eight times independently in the tribe. Abundant mucilage is s synapomorphy in *Lophomyrtus*+*Neomyrtus* (New Zealand clade) and the South American *Calyptranthes* (Fig. 7.11). Abundance of tannins was examined in the phloem of flowers (character 79), since this character has been cited as taxonomically useful to differentiate Australasian from South American genera of Myrtaceae (Schmid, 1972). Scarce content of tannins in flowers is the ancestral state for the tribe Myrteae and several genera (e.g., *Psidium*, *Myrceugenia*). Abundant tannins in phloem of flowers is a synapomorphy in the *Luma* + *Blepharocalyx* clade, which supports this phylogenetic relationship (Fig. 7.12). Abundant tannins in flowers is also a synapomorphy for the Chilean clade *Amomyrtus* + *Legrandia* and for the *Lophomyrtus* + *Neomyrtus* + *Lenwebbia* clade.

7.3.6 Biogeography

Parsimony reconstruction of biogeographic areas supports a Gondwanan origin of Myrteae, with Australasia optimized as the ancestral area of origin (Fig 7.13). The ancestral area of the family Myrtaceae and the tribe Myrteae has been optimized as Australia in previous investigations, but a possible South American, African or Antarctic origin has not been excluded (Sytsma et al., 2004; Thornhill et al., 2015). Although *Myrtus* (Mediterranean and North-Africa) might be interpreted as a LDD from Australasia (Fig. 7.13), the biogeographic history of the genus is uncertain in this analysis. It is unclear whether the divergence of *Myrtus* from all other extant Myrteae is a product of a LDD (as suggested in this investigation) or vicariance from an ancient and extinct Laurasian clade. The proposed break-up age between *Myrtus* and all other Myrteae has been estimated as 51 mya, which supports the LDD hypothesis, as this age is too recent to be product of a Gondwanan-Laurasian vicariance (Sytsma et al., 2004; Wilson, 2011; Thornhill et al., 2015). The presence of *Paleomyrtinea princetonensis* in North America has been explained as a product of a past-extended range of the tribe during the Paleocene and Eocene (Lucas et al., 2007).

The reconstructed ancestral area for Australasian Myrteae (e.g., *Decaspermum*, *Lithomyrtus, Rhodamnia, Gossia*) is unequivocally Australia (Fig. 7.13). Southern South America was reconstructed as the ancestral area for the NZ+*Lenwebbia+Ugni+Myrteola* clade. The strongly supported position (PP 1.0, BS 98) of *Lenwebbia* (Australia) sister to the New Zealand clade (*Lophomyrtus+Neomyrtus*) changes what was known for the New Zealand group, which was proposed as a direct LDD from South America (Lucas et al., 2007). The most recent common ancestor of the New Zealand genera (*Lophomyrtus* and *Neomyrtus*) *Lenwebbia* (Australia) is equivocal in the analyses, since it might be Australia or New Zealand. The explanation might be either a LDD from South America to Eastern Australia (*Lenwebbia*) with subsequent LDD to New Zealand (*Lophomyrtus+Neomyrtus*) or a LDE from South America to New Zealand with later dispersal to Eastern Australia. A LDD from South America to New Zealand might also explain the close relationship between *Myrteola* and the *New Zealand Lophomyrtus* and *Neomyrtus.*

All LDD events in the tribe (except for *Myrtus*) have been proposed to occur after the Late Eocene (Thornhill et al., 2015). The presence of *Metrosideros stipularis* exclusively in Southern South America is likely to be product of LDD from Australasia into South America (Figs 6.7, 6.8). Colonization of New Zealand by extant species has been proposed after the

re-emergence of lands in the late Oligocene. However, current fossil work is going to confirm crown ages of either extant or extinct New Zealand lineages, including *Metrosideros* and other Myrtaceae (Thornhill, pers. comm.).

The reconstructed ancestral area for South American Myrteae is southern South America (Chile-Argentina). The biogeographic reconstruction implies the dispersal of the tribe Myrteae from southern South America to the rest of South America (Fig. 7.13). It is likely that the ancestral area of Myrteae in southern South America was Chile, since most of the genera (including endemic monotypic and bitypic genera) are restricted to this country. Results suggest independent dispersal events from southern South America into North-Central America (*Pimenta* spp.) and the Juan Fernandez Islands (*Ugni selkirkii*, *Myrceugenia schulzei*, *Nothomyrcia fernandeziana*). Two of the three Juan Fernandez species (*Ugni selkirkii* and *Myrceugenia schulzei*) are sister to Chilean endemic species. The presence of some species of *Eugenia* and *Calyptranthes* that exclusively occur in North and Central America, are likely to be product of dispersal from South America.

Phylogenetic relationships presented in this investigation placed *M. coquimbensis* sister to all other *Myrcianthes* with very strong support. The simplest explanation for the isolated presence of *Myrcianthes coquimbensis* in the western coast of Chile is that the species might be product of a dispersal event from Eastern South America (Fig 7.13). Another more complex scenario is the possibility of a more widespread distribution of the ancestors of *Myrcianthes* across Eastern and Southern America and *M. coquimbensis* product of extinction of other lineages in the north of Chile due to changes in the geoclimatic conditions (Moreira-Muñoz, 2011). The presence of *Myrcianthes coquimbensis* only in a restricted and narrow distribution along the coast might be product of the migration of the species from increasingly dry areas to habitats with ocean breeze.

An unclear result from this investigation is the historical biogeography of *Nothomyrcia fernandeziana* (Juan Fernandez Islands). In this study, *Nothomyrcia fernandeziana* was phylogenetically nested with *Siphoneugena* and *Plinia* (Caribbean and eastern South America), which complicates the interpretation of such distant relationship in geographic terms. Other studies have recovered *Nothomyrcia* closely related to the Chilean *Blepharocalyx cruckshanksii* but with low-moderate support (Murillo et al., 2013). The phylogenetic scenario described in Murillo et al. (2013) might explain the extant presence of *Nothomyrcia fernandeziana* in the Juan Fernandez Archipelago.

The reconstructed ancestral area of *Myrceugenia* is southern South America, possibly Chile since most of the *Myrceugenia* species in southern South America occur in central-southern Chile and with no endemic *Myrceugenia* species in Argentina (Fig. 7.13). This result has been also proposed by previous studies (Murillo et al., 2012). From here, dispersal events led to the presence of the genus in Eastern South America (mainly Brazil) and the presence of *Myrceugenia chrysocarpa*, *Myrceugenia planipes*, *Myrceugenia ovata var. nanophylla* and *Myrceugenia exsucca* in southern Argentina. The biogeographical analysis supports the phylogenetic relationship obtained here for *Myrceugenia ovata. var. nanophylla* and *Myrceugenia exsucca*, both species occurring in the same biogeographic area (Southern South America). The presence of *Myrceugenia schulzei* in the Juan Fernandez Islands might be product of a LDD from Chilean *Myrceugenia* species, since it is closely related to *Myrceugenia colchaguensis* with strong support in this investigation and previous analyses (Murillo et al., 2012, 2013, 2016).

In this investigation, a very close phylogenetic relationship between *Ugni candollei* (restricted distribution in the coast of southern Chile) (Appendix 3) and *Ugni selkirkii* (Juan Fernandez Islands) was indicated. The sister position of *Ugni candollei* + *Ugni selkirkii* to all Myrteae in the parsimony total evidence tree (Fig. 6.5) suggests that these species might be early lineages of Myrteae in southern South America, probably from the same ancestors of the "*Myrteola* group" (*Ugni molinae* + *Ugni myricoides* + New Zealand genera + *Myrteola*). *Ugni selkirkii* in the Alejandro Selkirk Island is possibly due to a LDD from southern Chile. A close phylogenetic relationship between *Ugni myricoides* (widely distributed in America) and *Ugni molinae* (restricted to Southern Chile and Argentina) was also established in this investigation with strong statistical support. A biogeographic hypothesis for the extant distribution of *Ugni myricoides* and *Ugni molinae* might be that *Ugni molinae* was established as product of a dispersal event from northern South America, where *Ugni myricoides* is more abundant.

Luma and *Amomyrtus* have very similar biogeographic patterns, since both genera have two species each and one species is endemic to Central-South Chile and the other occurring in Central-South Chile and adjacent Argentina (Landrum, 1988). Both genera are monophyletic and have a South American most recent common ancestor. In this investigation, the ancestors of *Luma* were recovered as the same as *Myrceugenia* and *Blepharocalyx cruckshanksii* (Fig. 7.13). A possible explanation for the presence of *Luma apiculata* and *Amomyrtus luma* in Argentina might be the occurrence of dispersal events from Chile into Argentina, which is

similar to other genera of the Chilean-Argentinean flora (Arroyo, 2006; Moreira-Muñoz, 2011).

7.4 Conclusion

Herein, the evolution of 75 morphological characters related to leaves, flowers, fruits and secondary xylem and biogeographic areas were studied using ancestral state reconstruction. Results indicated that a few characters in Chilean species of Myrteae are ancestral and homologous with others observed in the outgroup (e.g., multiple epidermis in *Myrceugenia rufa* and *Metrosideros perforata*). Some morphological synapomorphies include dibrachiate hairs in *Myrceugenia*, anisocytic stomata in the *Lophomyrtus+Neomyrtus* clade and the genus *Austromyrtus* and a circular leaf vascular complex in *Amomyrtus, Calyptranthes* and *Luma*. An arc-shaped leaf vascular system is a clear synapomorphy for *Myrceugenia.* The presence of secretory cavities in anthers (character 68) (Fig. 7.9) reflects retention of the ancestral (plesiomorphic) character state in some groups, such as the Australasian clade (Australian + Asian species), the New Zealand clade (*Lophomyrtus* + *Neomyrtus* + *Lenwebbia*) and the *Luma+Blepharocalyx* clade. The absence of secretory cavities in anthers is apomorphic for a number of clades within Myrteae, but this character is highly homoplasious. A number of morphological characters have evolved independently several times during the evolutionary history of Myrteae independently (e.g., hypodermis, leaf marginal sclerenchyma). The exclusive presence of glandular hairs, dibrachiate hairs, spherical crystals and five layers of palisade parenchyma in the tribe Myrteae and its absence in the seven outgroup tribes, indicates that some characters may be evolutionary novelties in the family Myrtaceae. However, further investigation of such characters is necessary to confirm this hypothesis. Histochemical characters had interesting evolution patterns, with a number of synapomorphies within the tribe. Although many characters were optimized as synapomorphies in some groups, the evolution of several characters is highly homoplasious. The diversification of morphological characters involves character states that may confer ecological capabilities to some species, particularly those occurring in xerophytic habitats (e.g., *Myrceugenia rufa, Myrcianthes coquimbensis*). A number of character states potentially related to evolution in xerophytic habitats such as presence of hypodermis, multiple epidermis and straight epidermal anticlinal walls might be product of convergent evolution. Incorporation of new characters and the use of additional methods to reconstruct ancestral character states may improve the understanding of the morphological evolution in Myrteae.

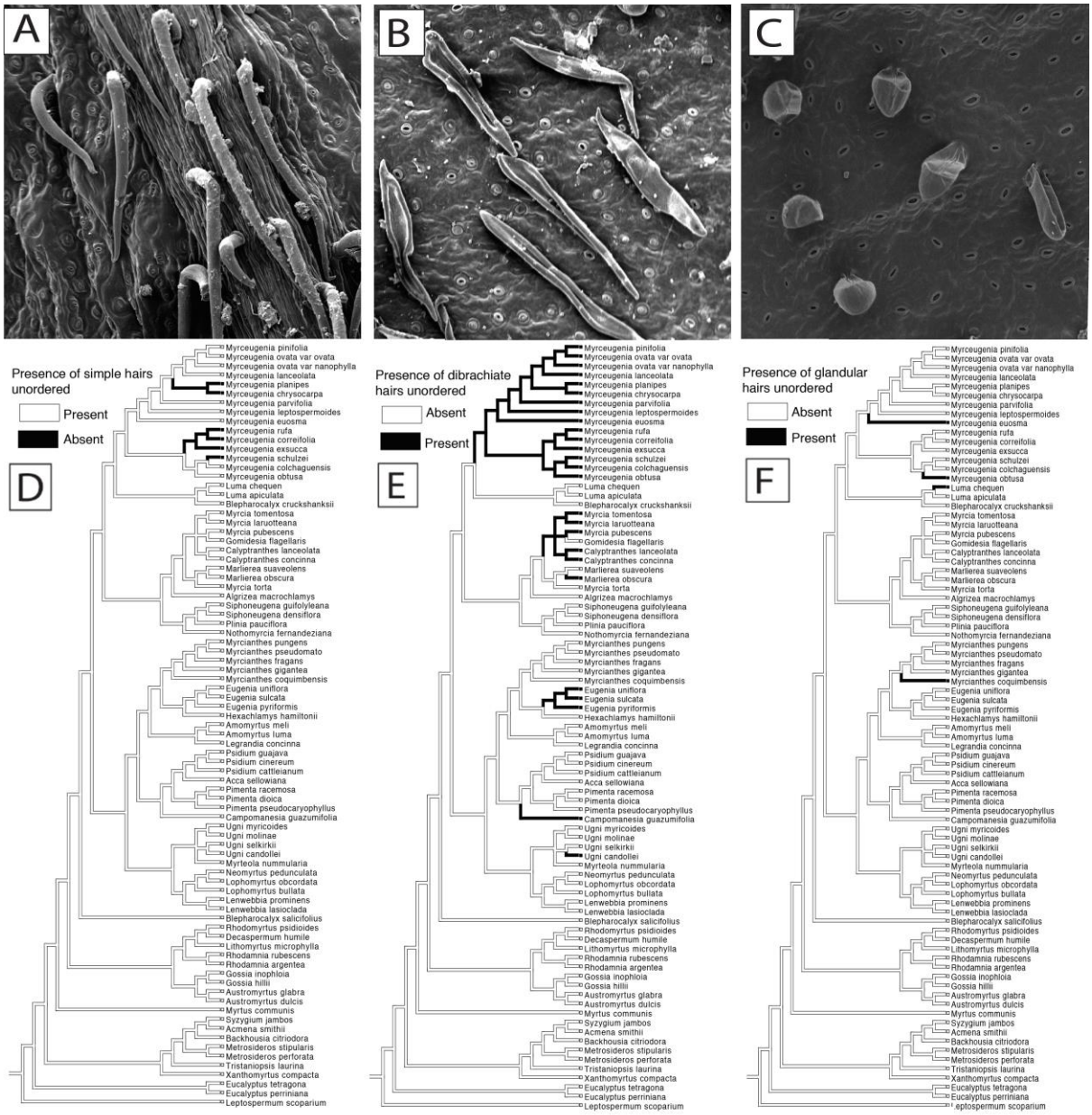


Figure 7.1. Type of hairs. A, simple hairs on the midrib of *Austromyrtus glabra*. B, dibrachiate hairs on the leaf blade of *Myrceugenia correifolia*. C, glandular hairs on the leaf blade of *Luma chequen*. D-F, ancestral state reconstruction of type of hairs in Myrteae: D, simple hairs. E, dibrachiate hairs. F, glandular hairs.

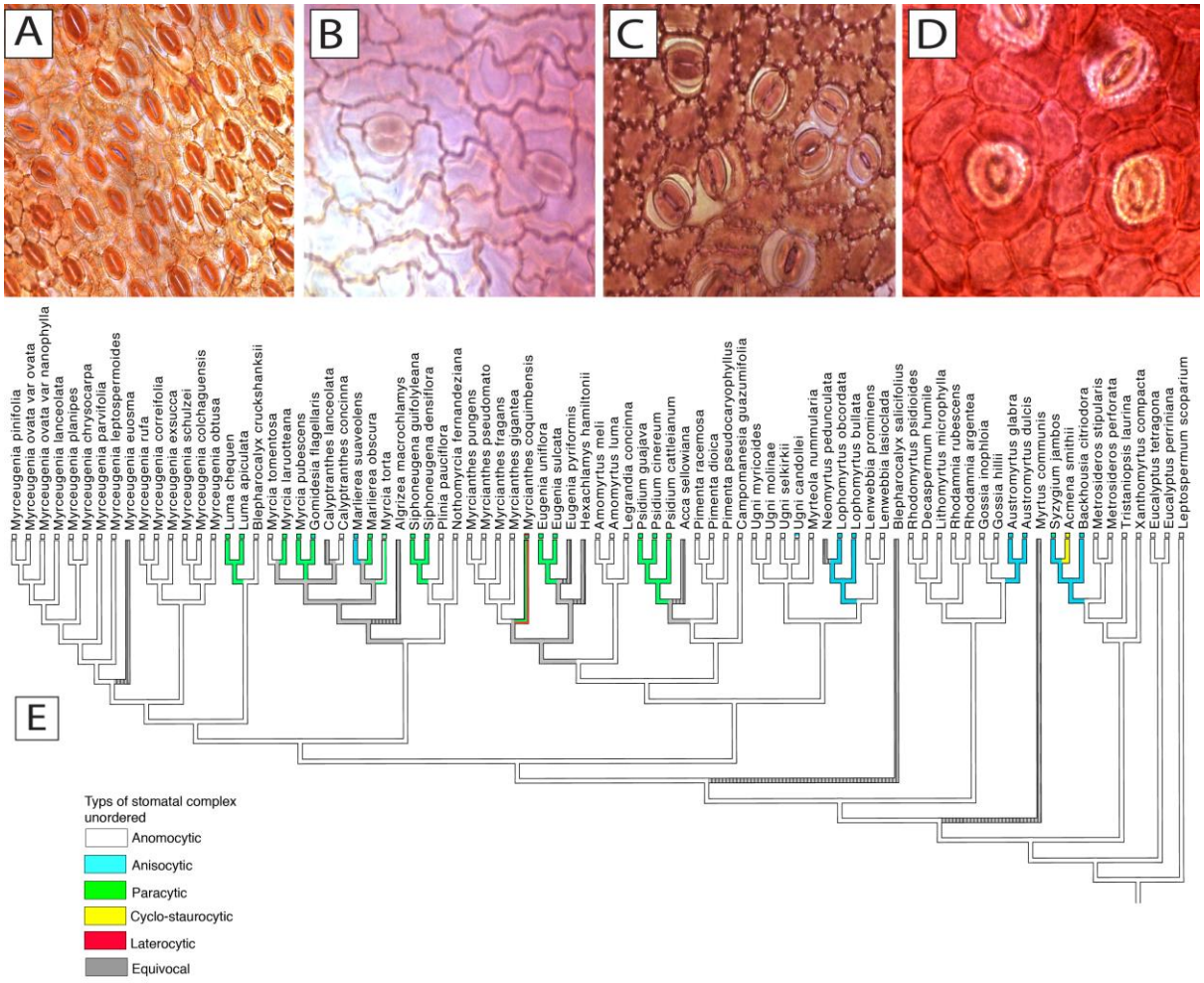


Figure 7.2. Types of stomatal complexes. A, anomocytic stomata in *Decaspermum humile*. B, anisocytic stomata in *Ugni candollei*. C, Paracytic stomata in *Psidium cinereum*. D, cyclo-staurocytic stomata in *Acmena smithii*. E, ancestral state reconstruction of types of stomata.

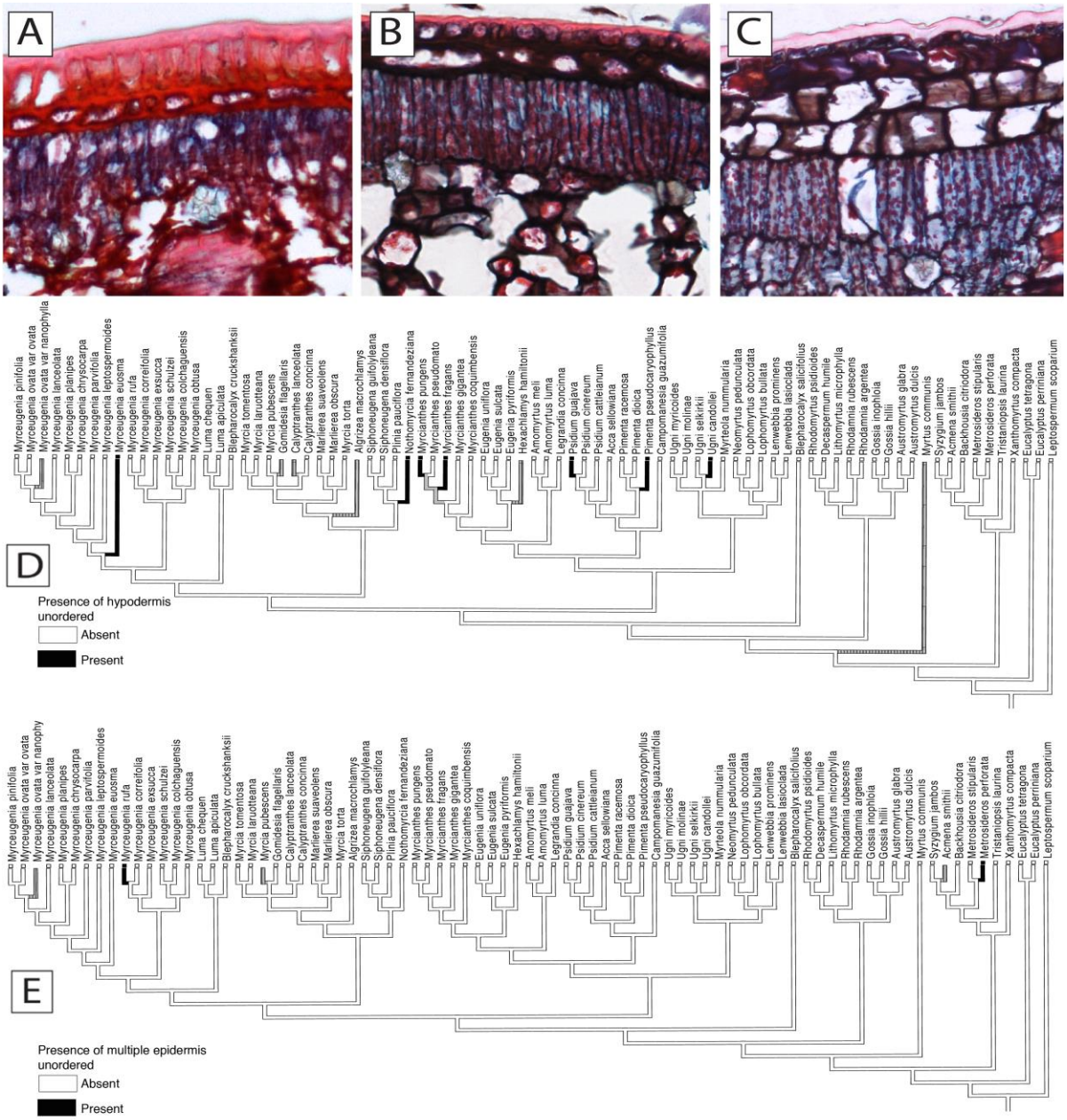


Figure 7.3. Adaxial hypodermis and multiple epidermis. A, hypodermis in leaves of *Pimenta pseudocaryophyllus*. B, hypodermis in leaves of *Myrcianthes pungens.* C, multiple epidermis in leaves of *Metrosideros perforata*. D, ancestral state reconstruction of hypodermis in Myrteae. E, ancestral state reconstruction of multiple epidermis in Myrteae.

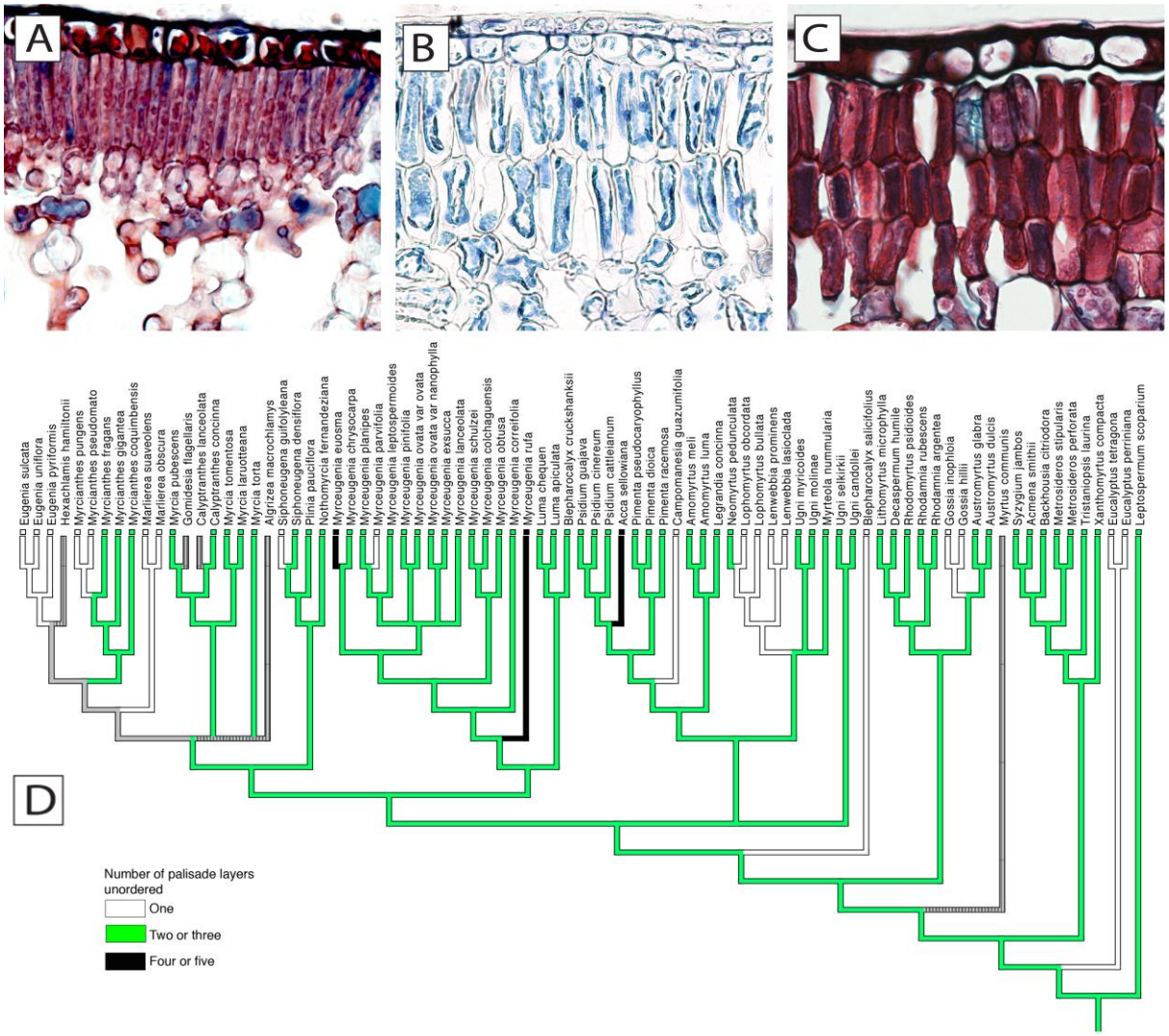


Figure 7.4. Number of palisade layers. A, one layer of palisade parenchyma in *Lenwebbia prominens*. B, two layers of palisade parenchyma in *Amomyrtus meli*. C, three layers of palisade parenchyma in *Legrandia concinna*. D, ancestral state reconstruction of number of palisade layers in Myrteae.

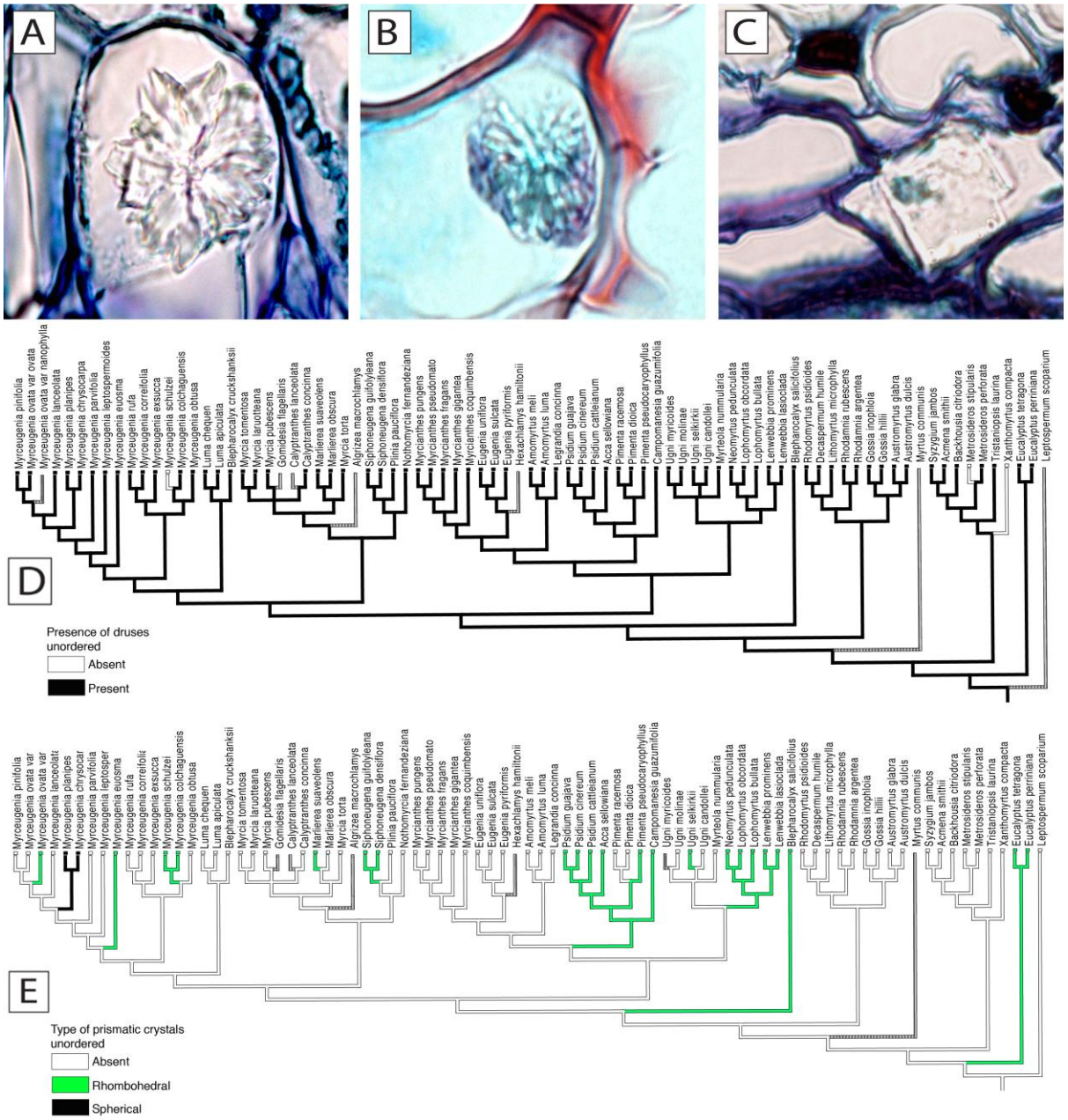


Figure 7.5. Presence of druses and prismatic crystals. A, Druse in *Amomyrtus luma*, B. prismatic spherical crystal in *Myrceugenia planipes*. C, prismatic rhombohedral crystal in *Ugni selkirkii*. D, ancestral state reconstruction of presence of druses in Myrteae. E, ancestral state reconstruction of type of prismatic crystals in Myrteae.

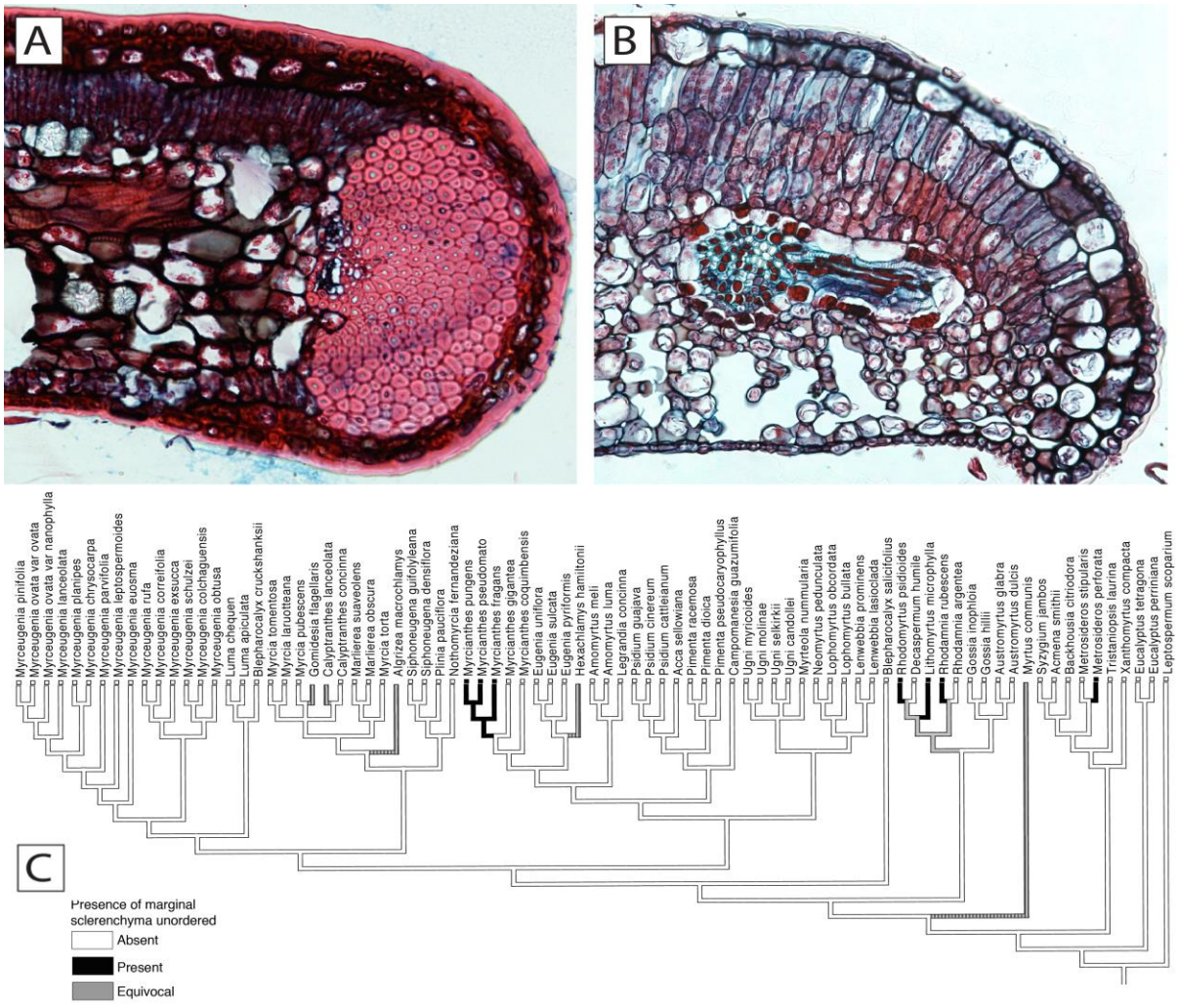


Figure 7.6. Presence of marginal sclerenchyma in leaves. A, marginal sclerenchyma present in *Myrcianthes pungens*. B, marginal sclerenchyma absent in *Ugni candollei*. C, ancestral state reconstruction of presence of marginal sclerenchyma in Myrteae.

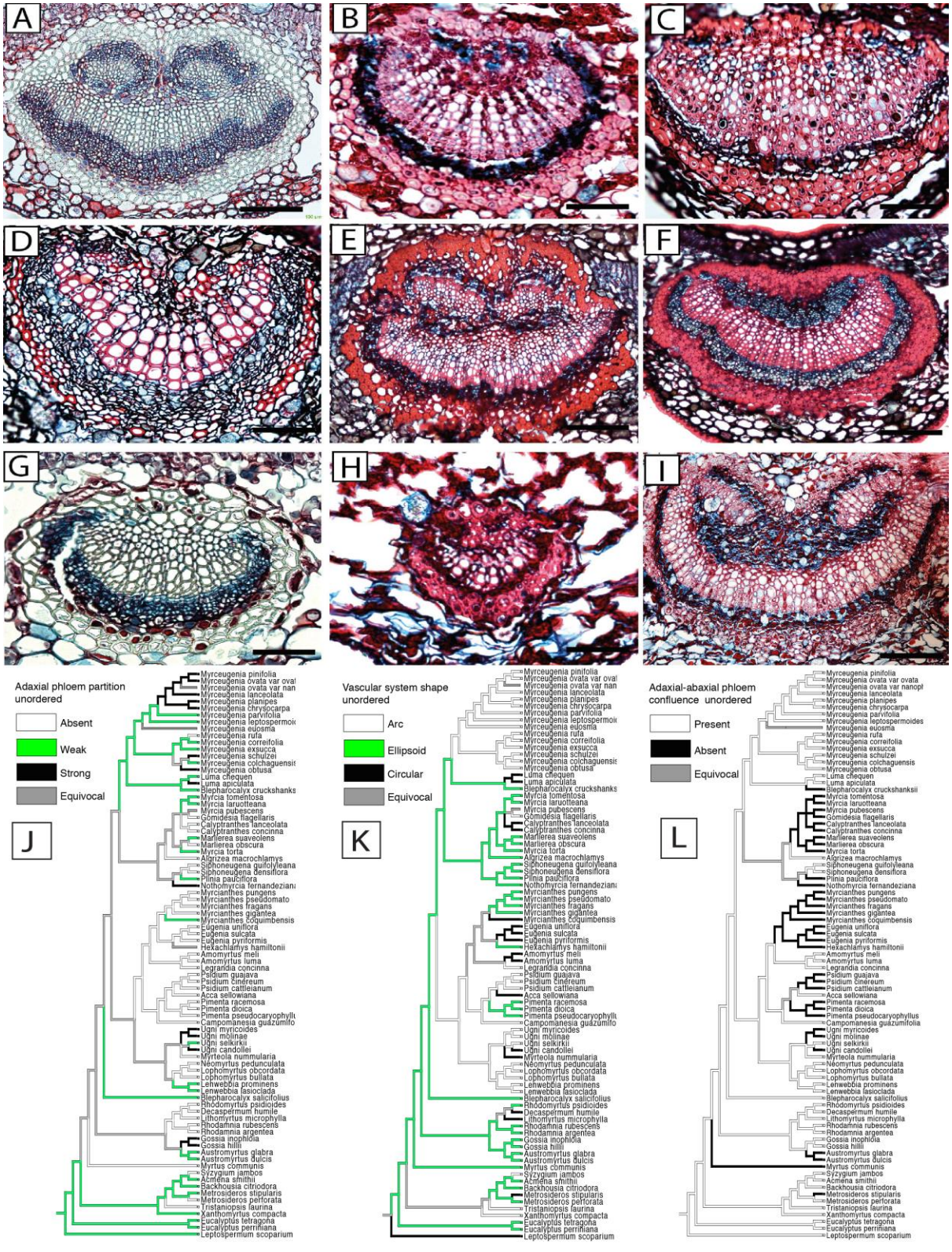


Figure 7.7. Leaf main vascular system. A, *Blepharocalyx cruckshanksii*. B, *Calyptranthes concinna*. C, *Gossia hillii*. D, *Lenwebbia lasioclada*. E, *Metrosideros perforata*. F, *Myrcianthes pungens*. G, *Myrceugenia obtusa*. H, *Neomyrtus pedunculata*. I, *Rhodamnia argentea*. J-L, ancestral state reconstruction of vascular complex characters: J, adaxial phloem partition. K, vascular system shape. L, adaxial-abaxial phloem confluence..

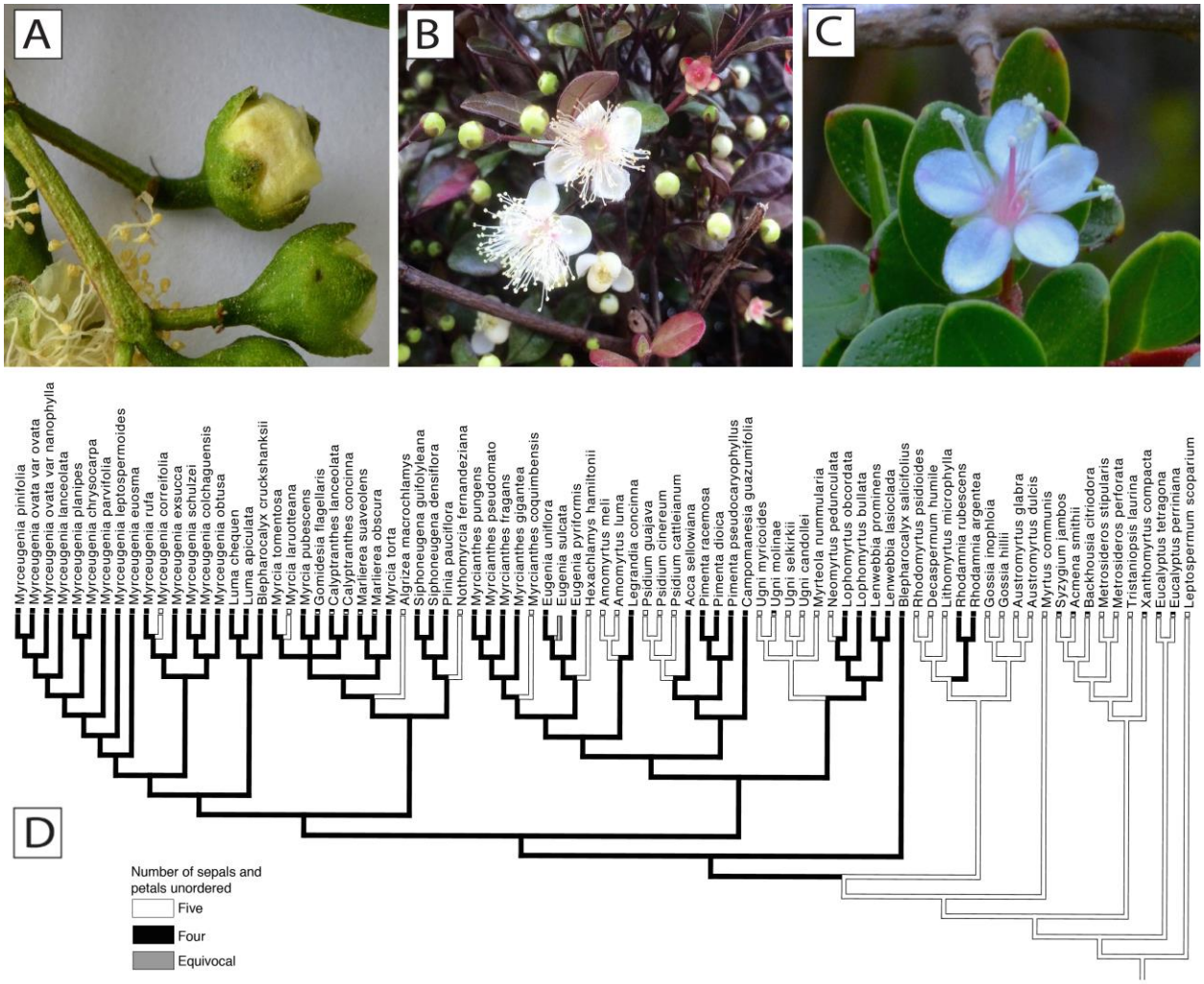


Figure 7.8. Number of sepals and petals. A, tetramerous flowers (four sepals and four petals) in *Blepharocalyx cruckshanksii*. B, tetramerous flowers in *Lophomyrtus bullata*. C, pentamerous (5 sepals and 5 petals) flowers in *Myrteola nummularia*. D, ancestral state reconstruction of number of sepals and petals in Myrteae

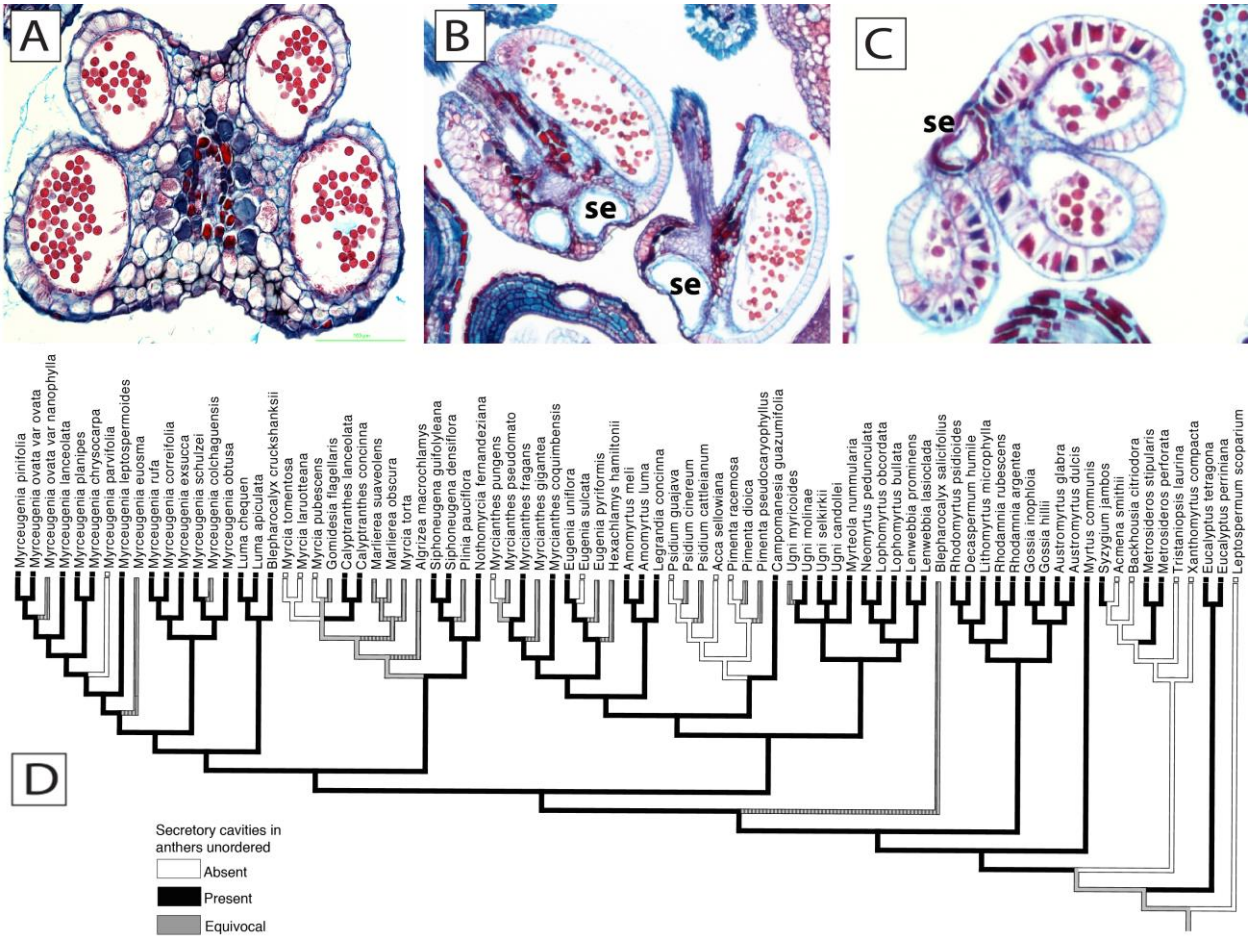


Figure 7.9. Secretory cavities in anthers. A, anther without secretory cavities in *Myrceugenia parvifolia*. B, anthers with secretory cavities in *Ugni candollei*. C, anthers with secretory cavities in *Metrosideros stipularis*. D, ancestral state reconstruction of secretory cavities in anthers in Myrteae. se: secretory cavities.

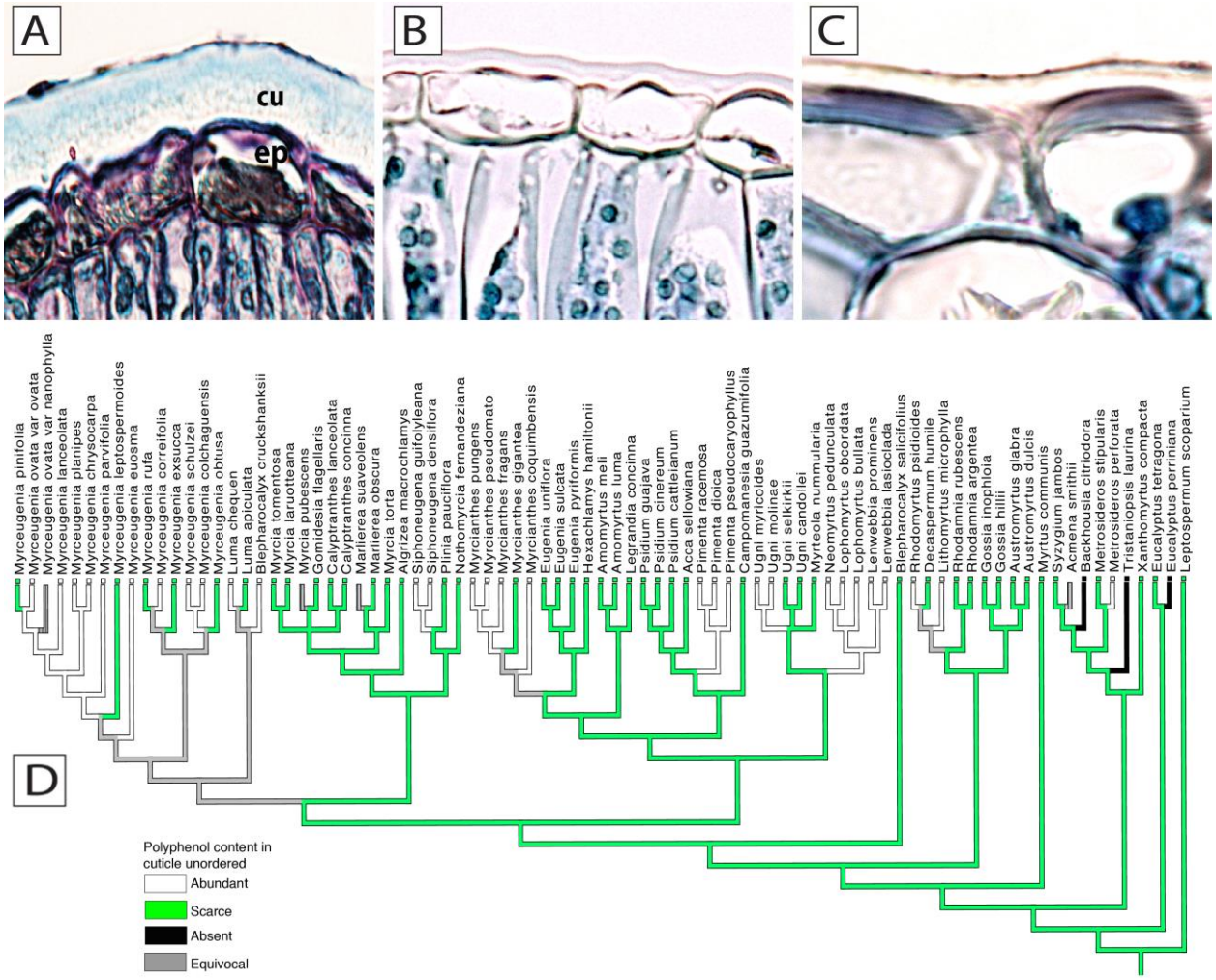


Figure 7. 10. Polyphenol content in cuticle. A, abundant polyphenols in the cuticle of *Ugni molinae* (strong blue staining). B, scarce polyphenol content in the cuticle of *Amomyrtus luma* (weak blue staining). C, absence of polyphenols in the cuticle of *Eucalyptus perriniana* (absence of blue staining). D, ancestral state reconstruction of polyphenol content in cuticle in Myrteae. cu: cuticle, ep: epidermis. Polyphenols stained blue in A.

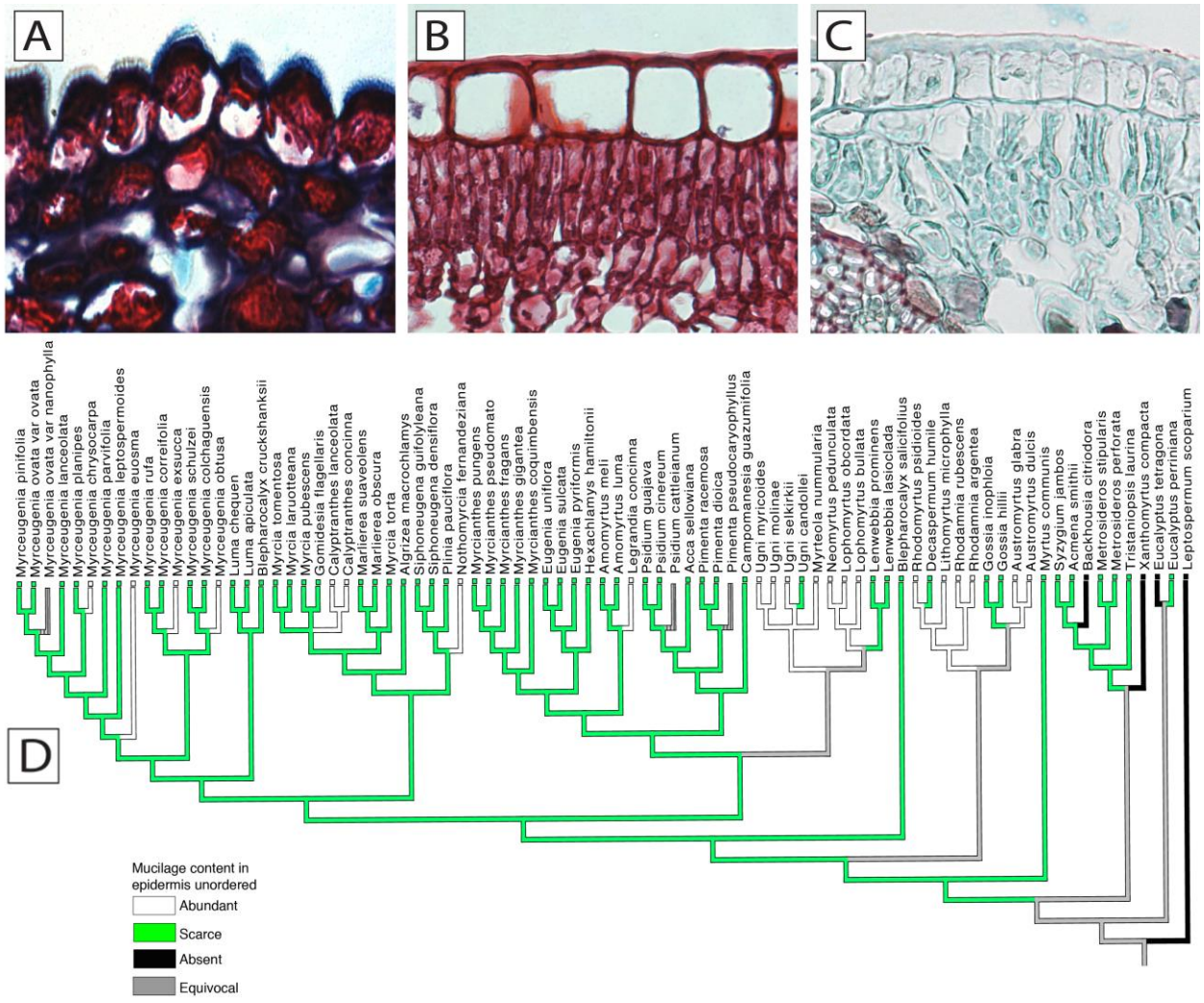


Figure 7. 11. Mucilage content in epidermis. A, abundant mucilage content in the epidermis of *Lithomyrthus microphylla*. B, scarce mucilage content in the epidermis of *Myrceugenia planipes*. C, mucilage absent in the epidermis of *Backhousia citriodora*. D, ancestral state reconstruction of mucilage content in epidermis. Mucilage stained red in A and B.

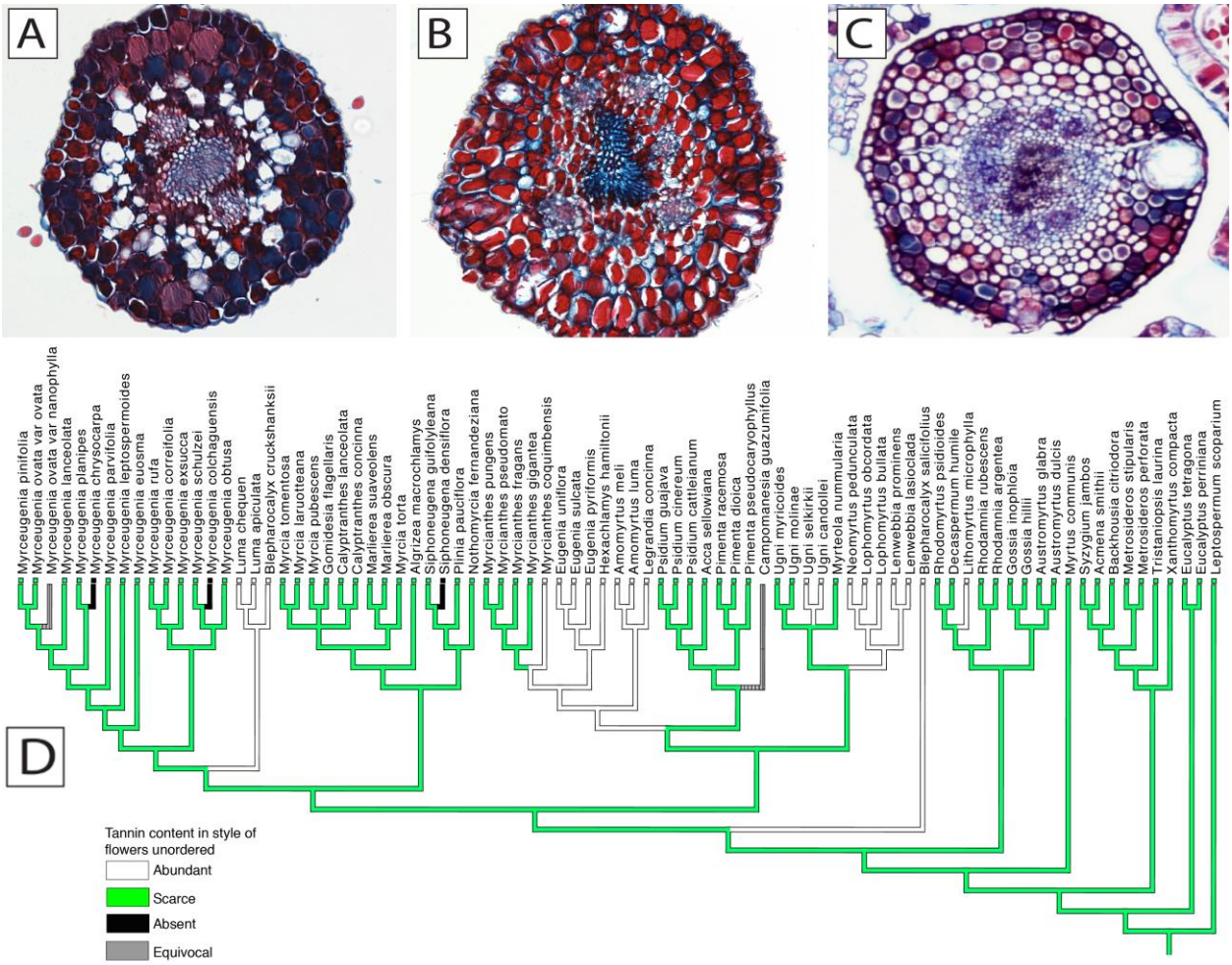


Figure 7.12. Tannin content in style of flowers. A, abundant tannin content in the style of *Luma apiculata*. B, abundant tannin content in the style of *Blepharocalyx cruckshanksii*. C, scarce tannin content in the style of *Myrceugenia planipes*. D, ancestral state reconstruction of tannin content in style of flowers. Tannins stained red-purplish in A, B and C.

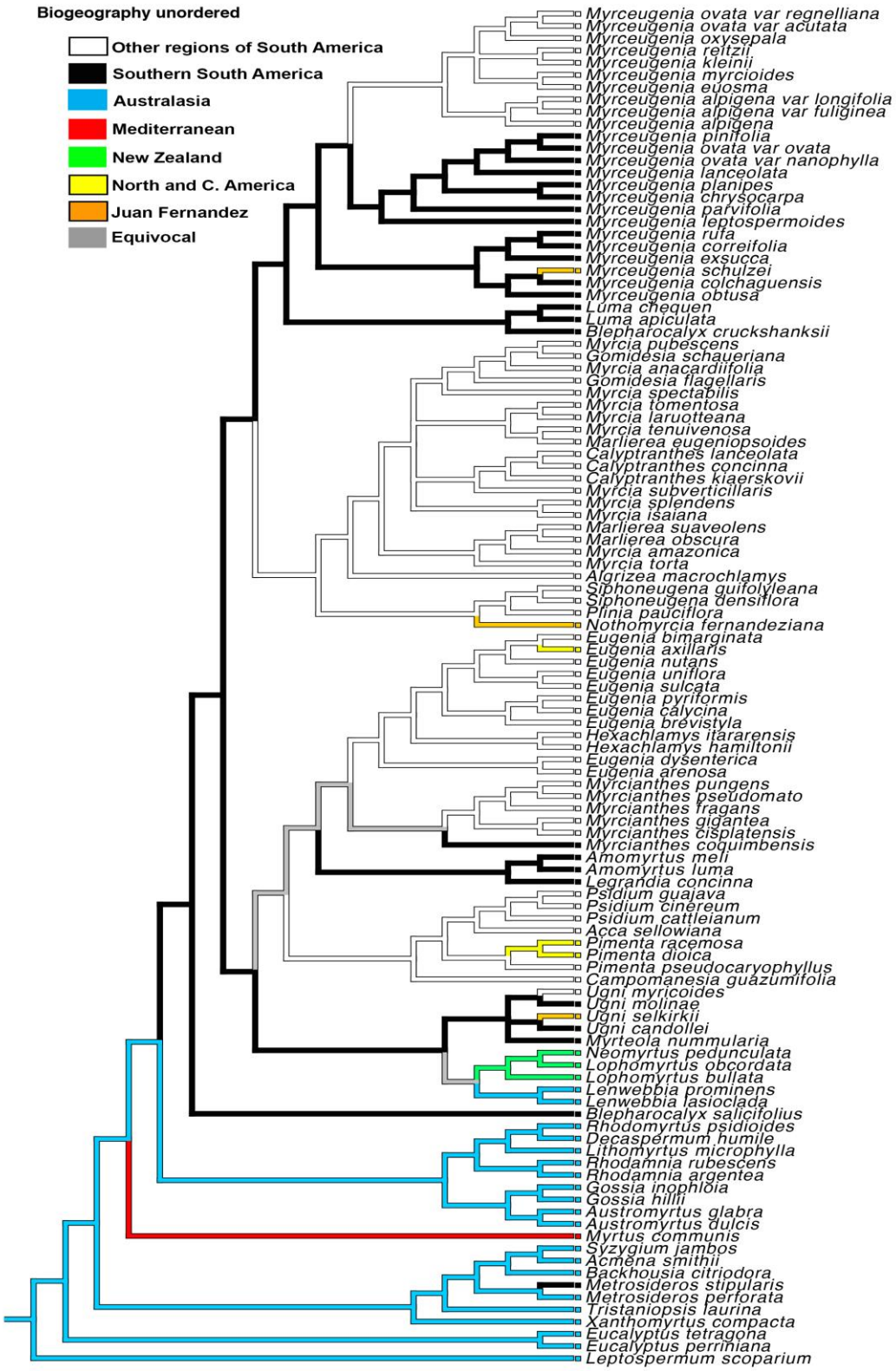
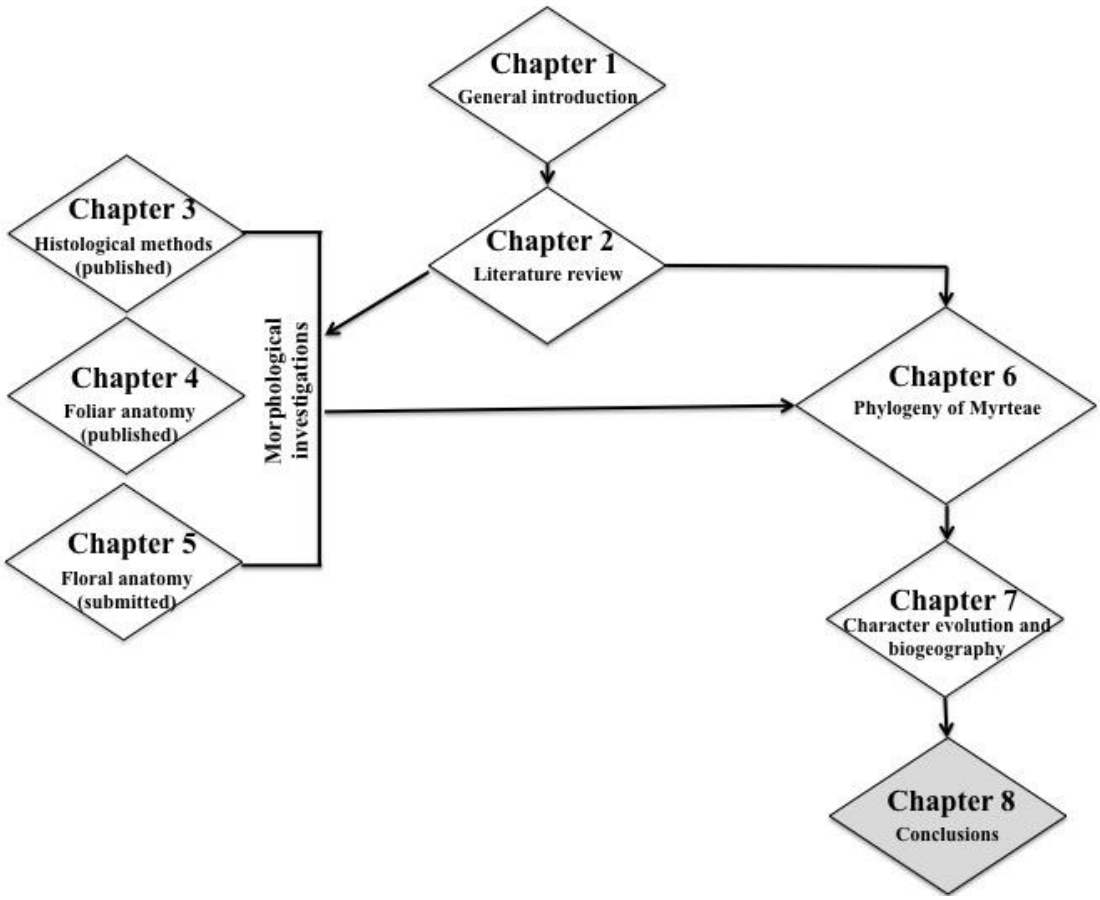


Figure 7.13. Majority rule tree obtained from Bayesian analysis of the total evidence data set (ETS, ITS, psbA-trnH, matK, morphology) with biogeography optimized as unordered multistate character. Biogeographic areas adapted from Good (1974) and Huggett (2004).


Chapter 1
General introduction
Chapter 2
Literature review
Chapter 3
Histological methods (published)
Chapter 4
Foliar anatomy (published)
Chapter 5
Floral anatomy (submitted)
Morphological investigations
Chapter 6
Phylogeny of Myrteae
Chapter 7
Character evolution and biogeography
Chapter 8
Conclusions

CHAPTER 8: Conclusions

8.1 Significance of the thesis and key findings

The purpose of the present study was to determine the systematic position of all species of Chilean Myrteae by conducting a tribal-level phylogenetic study using both molecular and morphological data. This research greatly extends the level of knowledge of floral and foliar morpho-anatomical characters in Myrteae and particularly so for the Chilean species.

The protocol for histochemical investigation developed during this research (Chapter 3) provides a standardised method for comparing staining reactions across the family. This thesis includes the first comprehensive anatomical, micromorphological and histochemical investigation of leaves and flowers in all the 26 species of Chilean Myrtaceae (25 Myrteae and one Metrosidereae) (Chapters 4 and 5). This investigation has generated DNA sequences of a number of species and genera for the first time (Chapter 6), namely *Myrcianthes coquimbensis*, *Ugni candollei*, *Ugni myricoides*, *Lenwebbia lasioclada*, *Lenwebbia prominens*, *Lithomyrtus microphylla* and *Austromyrtus glabra*. Several DNA sequences from new loci where generated for a number for species, particularly *psbA-trnH* for *Myrceugenia* (10 species). This study has generated extensive anatomical and micromorphological data for the tribe Myrteae, expanding the knowledge from few genera and species to most of the genera of the tribe. An anatomical/micromorphological identification key has been provided (Chapter 4) for nine genera of Myrteae that might be potentially useful to describe fossil material for calibrated phylogenies.

Phylogenetic analyses indicated that four Chilean genera (*Amomyrtus*, *Myrceugenia*, *Luma* and *Myrcianthes*) are monophyletic with strong statistical support. The other Chilean genera are either represented for one species (*Myrteola*), are monotypic (*Legrandia*) and not monophyletic but with low support (*Ugni*, *Blepharocalyx*). The species of Chilean Myrteae are distributed among six clades. *Legrandia* and *Amomyrtus* formed a well-supported group in most of the analyses. *Nothomyrcia* had unclear relationships, positioned with the Brazilian genus *Siphoneugena* in combined molecular analyses but with low support. The four *Ugni* species formed two well-supported monophyletic groups (*U. molinae* + *U. myricoides* and *U. candollei* + *U. selkirkii*), with unclear phylogenetic position. The four species of *Ugni* are likely to be nested within a clade formed by the New Zealand genera (*Lophomyrtus* and *Lophomyrtus*), *Myrteola nummularia* and the Australian genus *Lenwebbia*. However, MP

analyses of combined molecular and morphological data do not place all four species in the same clade as New Zealand and allied genera. For the first time, the four species of *Ugni* have been included in a single phylogenetic study. The two Chilean varieties of *Myrceugenia ovata* (*var. ovata* and *var. nanophylla*) were monophyletic in the morphological phylogeny but not in the molecular or total evidence analyses. *Blepharocalyx cruckshanksii* was sister to the genus *Luma* in a clade with *Myrceugenia* with high Bayesian support but low bootstrap support. *Myrcianthes coquimbensis* was as sister to all other species of *Myrcianthes* with high statistical support. The addition of morphological data, in particular floral and foliar characters, to phylogenetic analyses improved the resolution and statistical support for key nodes, something that has been lacking from all tribal level studies to date.

Character evolution analyses (Chapter 7) showed that morphological synapomorphies include dibrachiate hairs in the genus *Myrceugenia*, anisocytic stomatal complexes in *Lophomyrtus* + *Neomyrtus* and *Austromyrtus*, paracytic stomata in *Luma* and *Psidium* and an arc-shaped leaf vascular system in *Myrceugenia*. The presence of multiple epidermis in the two unrelated *Myrceugenia rufa* and *Metrosideros perforata* (outgroup) is apomorphic and independently derived (convergent evolution). Many morphological characters have evolved a number of times independently within Myrteae. Biogeographic analyses support a Gondwanan origin of Myrteae with *Myrtus* in the Mediterranean probably as product of a LDD. The morphological investigation indicates that most of the species of Myrteae have a mesophytic leaf anatomy and micromorphology. Some species such as *Myrceugenia rufa, Myrceugenia correifolia* and *Myrcianthes coquimbensis* have a number of characters associated to xerophytic habitats (e.g., adaxial hypodermis, straight anticlinal walls, and abundant hairs) that might be result of convergent evolution. *Myrcianthes coquimbensis* was previously known as a species in a monotypic genus (*Reichea coquimbensis*) (Kausel, 1940, 1942). Landrum and Grifo (1988) transferred *Reichea* to *Myrcianthes*, based on three morphological characters (perianth-mery, inflorescence and embryo). Due to extreme geographical separation from other species of the same genus, a strongly supported sister relationship to all the other *Myrcianthes*, a placenta slightly more protruding than the other species of *Myrcianthes* and its rare anatomical xerophytic condition, the reinstatement of *Reichea* might be needed. However, a more comprehensive phylogeny of *Myrcianthes* is recommended for a further investigation of the genus.

8.2 Future work and recommendations

Limitations of the current and previous investigations (e.g., Lucas et al., 2007; Murillo et al., 2012, 2013, 2016) have not allowed the reliable reconstruction of relationships in Myrteae with high statistical support. Bayesian total evidence analyses have provided a step forward towards a more resolved phylogeny of Myrteae. Improved resolution and statistical support might be achieved with the use of additional molecular loci and more informative morphological characters. The use of next-generation sequencing may be a useful approach to underpin a more robust phylogeny of the tribe Myrteae. The presence of secondary metabolites in Myrtaceae might be problematic when extracting and amplyfing DNA in some species. For this reason, it is recommended to optimize a DNA extraction-amplification protocol for Myrtaceae, similar to the staining protocol developed in this thesis. It is strongly recommended to improve the taxon sampling of Myrteae and include a number of poorly known species such as *Curitiba prismatica, Amomyrtella guili, Calycolpus* spp. and *Meteoromyrtus* (*Eugenia*) *wynadensis* (recently revised by Wilson and Heslewood, 2016). In addition, species included in this investigation with a broad distribution (e.g., *Ugni myricoides*, *Myrteola nummularia*, South America) should be investigated using multiple accessions. Similarly, more accessions are needed for a deeper investigation on the *Myrceugenia ovata* complex including the two Brazilian and the two Chilean species. A more comprehensive phylogeny including more species of *Myrcianthes* and the species *Blepharocalyx eggersii* might provide a clearer scenario regarding the evolution of these two genera.

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Appendices

Appendix 1. Voucher information for species used for morphological and molecular work with taxonomic authorities and geographic distribution. Number of species in each genera based on Wilson (2011). CONC: University of Concepcion Herbarium. EIF: Forestry Herbarium of the University of Chile. NSW: National Herbarium of New South Wales. BRI: Queensland Herbarium. Species not included in this list were not studied with vouchers and information was taken from the literature (including Genbank, NCBI, 2016).

Genera (approx. number of species)	Species included in this study	Main Distribution	Voucher
	Tribe Backhousieae		
Backhousia (8)	*Backhousia citriodora* F.Muell.	Australia	A.R.Bean 16303-D.Fawett 1985 (BRI)
	Tribe Eucalypteae		
Eucalyptus (800)	*Eucalyptus perriniana* F.Muell. ex Rodway	Australia	H.Retamales 01388 (BRI)
	Tribe Kanieae		
Tristianopsis (40)	*Tristaniopsis laurina* (Sm.) Peter G.Wilson & J.T.Waterh.	Australia	G.N. Batianoff 030734 (BRI)
	Tribe Metrosidereae		
Metrosideros (30)	*Metrosideros perforata* (J.R.Forst. & G.Forst.) Druce	Australia	J.M. Lecussan 961 (BRI)
	Metrosideros (*Tepualia*) *stipularis* (Hook. & Arn.) Hook.f.	Chile and Argentina	H.Retamales 11805 (EIF)
	Tribe Myrteae		
Amomyrtus (2)	*Amomyrtus luma* (Mol.) D.Legr & Kaus.	Chile-Argentina	H.Retamales 11819-11820 (EIF)
	Amomyrtus meli (Phil.) D.Legr. & Kaus.	Chile	H.Retamales 11789-11799 (EIF)
Austromyrtus (3)	*Austromyrtus dulcis* (C.T.White) L.S.Sm.	Australia	P.I.Forster 38473 (BRI)

Genera (approx. number of species)	Species included in this study	Main Distribution	Voucher
	Austromyrtus glabra N. Snow & Guymer	Australia	J. Halford 792 (BRI)
Blepharocalyx (3)	*Blepharocalyx cruckshanksii* (Hook. & Arn.) Nied.	Chile	H.Retamales 11803 (EIF)
Calyptranthes (100)	*Calyptranthes concinna* DC.	Brazil	Zardini y Velazquez 23940 (NSW)
	Calyptranthes lanceolata O.Berg	Brazil	Wasum 1239 (NSW)
Campomanesia (70)	*Campomanesia guazumifolia* (Cambess.) O.Berg	Brazil	Eiten and Liene 10021 (BRI)
Decaspermum (30)	*Decaspermum humile* (Sweet ex G. Don) A.J. Scott	Australia and South-East Asia	H.Retamales 029.1-2 (BRI)
Eugenia (550)	*Eugenia uniflora* O.Berg	Brazil	C.J. Nicholson 35A (BRI)
Gomidesia (60)	*Gomidesia flagellaris* (D.Legrand) Sobral	Brazil	Sobral 5066 (NSW)
Gossia (30)	*Gossia hillii* (Benth.) N.Snow & Guymer	Australia	Hyland 25394-J.Halford 782 (BRI)
	Gossia inophloia (J.Bailey & C.White) Snow and Guymer	Australia and South-East Asia	A. Ford 02029-Gleiper 2012 (BRI)
Legrandia (1)	*Legrandia concinna* (Phil.) Kausel	Chile	H.Retamales 11821 (EIF)
Lenwebbia (2)	*Lenwebbia lasioclada* (F. Muell.) N.Snow & Guymer	Australia	P.I. Forster 17723 (BRI)
	Lenwebbia prominens N.Snow & Guymer	Australia	N.Nicholson 2393 (BRI)
Lithomyrtus (11)	*Lithomyrtus microphylla* (Benth.) N.Snow & Guymer	Australia	A.Ford and B.Hewett (BRI)
Lophomyrtus (2)	*Lophomyrtus bullata* Burret	New Zealand	W.R. Sykes 42 (NSW)
	Lophomyrtus obcordata (Raoul) Burret	New Zealand	Rogarder 757 (NSW)
Luma (2)	*Luma apiculata* (DC.) Burret	Chile-Argentina	H.Retamales 11811 (EIF)
	Luma chequen (Mol.) A.Gray	Chile	H.Retamales 11812 (EIF)
Marlierea (100)	*Marlierea obsura* O.Berg	Brazil	Guillespie 2687 (NSW)
Myrceugenia (40)	*Myrceugenia chrysocarpa* (O.Berg) Kausel	Chile-Argentina	H.Retamales 11796 (EIF)
	Myrceugenia colchaguensis (Phil.) Navas	Chile	Crawford and Baeza 116898 (CONC)

Genera (approx. number of species)	Species included in this study	Main Distribution	Voucher
	Myrceugenia correifolia (Hook. & Arn.) O.Berg	Chile	H.Retamales 11807 (EIF)
	Myrceugenia exsucca (DC.) O.Berg	Chile-Argentina	H.Retamales 11815 (EIF)
	Myrceugenia lanceolata (Juss. ex J. St.-Hil.) Kausel	Chile	H.Retamales 11818 (EIF)
	Myrceugenia leptospermoides (DC.) Kausel	Chile	H.Retamales 11821 (EIF)
	Myrceugenia obtusa (DC.) O.Berg	Chile	H.Retamales 11800 (EIF)
	Myrceugenia ovata var. ovata (Hook. & Arn.) O.Berg	Chile	H.Retamales 11801 (EIF)
	Myrceugenia ovata var. nanophylla (Burret) L.R. Landrum	Chile-Argentina	Crawford and Baeza 157851 (CONC)
	Myrceugenia parvifolia (DC.) Kausel	Chile-Argentina	H.Retamales 11810 (EIF)
	Myrceugenia pinifolia (F. Phil.) Kausel	Chile	Crawford and Baeza 157850 (CONC)
	Myrceugenia planipes (Hook. & Arn.) O.Berg	Chile-Argentina	H.Retamales 11802 (EIF)
	Myrceugenia rufa (Colla) Skottsb. ex Kausel	Chile	H.Retamales 11813 (EIF)
	Myrceugenia schulzei Johow	Juan Fernandez Island	H.Retamales 11814 (EIF)
Myrcia (500)	*Myrcia laruotteana* Cambess.	Brazil	Zardini 8638 (NSW)
	Myrcia tomentosa (Aubl.) DC.	Brazil	Proenca 942 (NSW)
	Myrcia torta DC.	Brazil	Zardini 8633 (NSW)
Myrcianthes (30)	*Myrcianthes coquimbensis* (Barnéoud) Landrum & Grifo	Chile	H.Retamales 11822 (EIF)
	Myrcianthes fragrans (Sw.) McVaugh	Florida, Mexico to Peru	Zanoni, Mejia and Pimentel 18075 (NSW)
	Myrcianthes pungens (O. Berg) D. Legrand	Brazil, Paraguay to Argentina	Grifo 547 (NSW)
Myrteola (3)	*Myrteola nummularia* (Poir.) O.Berg	Chile-Argentina-Peru-Bolivia-Ecuador-Venezuela-Colombia	H.Retamales 11804 (EIF)
Neomyrtus (1)	*Neomyrtus pedunculata* (Hook. f.) Burret	New Zealand	Rogarder 981-Brownlie 575 (NSW)

Genera (approx. number of species)	Species included in this study	Main Distribution	Voucher
Nothomyrcia (1)	*Nothomyrcia fernandeziana* (Hook. & Arn.) Kausel	Juan Fernandez Islands	H.Retamales 11816 (EIF)
Pimenta (15)	*Pimenta racemosa* (Mill.) J.W. Moore	Central America	H.Retamales 093 (BRI)
Plinia (40)	*Plinia pauciflora* M.L. Kawas & B. Holst	Brazil	Zardini 23970 (NSW)
Psidium (20)	*Psidium guajava* L.	Central America to Brazil	I.G. Champion 1148-J. Kemp 17044 (BRI)
Rhodamnia (5)	*Rhodamnia argentea* Benth.	Australia	P.I.Forster 28065-J.Halford 783 (BRI)
	Rhodamnia rubescens Benth.	Australia	J. Halford 783-P.I. Forster 28065 (BRI)
Rhodomyrtus (18)	*Rhodomyrtus psidioides* (G.Don) Benth.	Australia	P. Grimshaw 2911-A.R. Bean 28097 (BRI)
Siphoneugena (8)	*Siphoneugena densiflora* O.Berg	Brazil	Proenca 407 (NSW)
Ugni (4)	*Ugni candollei* (Barnéoud) O.Berg	Chile	H.Retamales 11806 (EIF)
	Ugni molinae Turcz.	Chile-Argentina	H.Retamales 11808 (EIF)
	Ugni myricoides (Kunth) O.Berg	North America-Colombia-Venezuela-Brazil-Peru	H.Retamales 11809 (EIF)
	Ugni selkirkii (Hook. & Arn.) O.Berg	Juan Fernandez Islands	T.Stuessy and Crawford 121491 (CONC)
	Tribe Syzygieae		
Acmena (15)	*Acmena* (*Syzygium*) *smithii* (Poir.) Merr. & L. M. Perry	Australia	H.Retamales 032.1-2 (BRI)
Syzygium (500)	*Syzygium (Waterhousea) floribundum* F.Muell.	Australia	H.Retamales 030.1-2 (BRI)
	Tribe Tristanieae		
Xanthomyrtus	*Xanthomyrtus compacta* Diels	New Guinea	W.R. Barker 66951 (BRI)

Appendix 2. Character definitions and references consulted for phylogenetic reconstruction and character evolution. Characters and character states based on Landrum (1981), Johnson and Briggs (1984), Wilson et al. (2001), Lucas et al. (2007), Soh and Parnell (2011), Pimentel et al. (2014) and Vasconcelos et al. (2015) are referenced as L#, J&B#, W#, LL#, S&P#, P# and V# respectively, where # is the number of character in the original investigation if applicable. Characters obtained for other sources are referred to where relevant. Characters newly constructed for this study are indicated by *.

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1. Habit: (0) tree, (1) erect /rounded shrub, (2) creeping shrub (J&B1, character states modified following Landrum, 1988a).

2. Flowering period: (0) late winter and spring, (1) summer and fall (J&B, defined in the publication but not numbered).

3. Vegetative phyllotaxy: (0) opposite, (1) alternate, (2) verticillate (W15, J&B25, 26, S&P17, character states modified following Wilson, 2011).

4. Abaxial leaf pubescence: (0) dense, (1) sparse or lacking (L22).

5*. Type of primary venation: (0) pinnate, (1) basal.

6*. Conspicuous secondary veins: (0) inconspicuous, (1) weakly conspicuous, (2) strongly conspicuous.

7. Type of foliar colleters: (0) conic, (1) euryform, (2) petaloid, (3) absent (Da Silva et al., 2012).

8. Presence of simple hairs on leaves: (0) present, (1) absent (L24 and own observations).

9. Presence of dibrachiate hairs on leaves: (0) present, (1) absent (L24 and own observations).

10*. Presence of glandular hairs on leaves: (0) absent, (1) present.

11*. Cuticular thickness: (0) less than 6 μm, (1) 6 μm or more.

12. Lamina cuticular ornamentation: (0) smooth, (1) striate (Fontenelle et al., 1994, Haron and Moore, 1996).

13. Type of stomatal complex: (0) anomocytic, (1) anisocytic, (2) paracytic, (3) cyclo-staurocytic, (4) laterocytic (S&P1).

14. Stomatal level: (0) level, (1) sunken (Haron and Moore, 1986).

15*. Distribution of stomata: (0) abaxial surface, (1) abaxial and adaxial surfaces.

16. Adaxial epidermis cell shape (cross-section): (0) isodiametric, (1) wider than high, (2) higher than wide (S&P10).

17. Abaxial epidermis cell shape (cross-section): (0) isodiametric, (1) wider than high, (2) irregular (S&P11).

18. Adaxial hypodermis: (0) absent, (1) present (Cardoso et al., 2009, Retamales and Scharaschkin, 2015).

19. Multiple epidermis: (0) absent, (1) present (Retamales and Scharaschkin, 2015)

20. Epidermis abaxial anticlinal cell wall: (0) slightly sinuous, (1) highly sinuous, (2) straight (S&P2, Fontenelle et al., 1994 for South American Myrteae).

21. Conical papillae on leaves: (0) absent, (1) present (Cardoso et al., 2009, Retamales and Scharaschkin, 2015).

22. Mesophyll structure: (0) dorsiventral, (1) isobilateral (S&P13, originally called "abaxial palisade layers").

23. Number of palisade parenchyma layers: (0) one, (1) two or three, (2) four or five (Cardoso et al., 2009 for South American Myrteae).

24. Druses: (0) absent, (1) present (S&P4, following arguments in Schmid, 1980, we have added the absent state (3) to S&P4. Soh and Parnell, 2011 included druses and prismatic crystals in one character. In the present study, druses and prismatic crystals were split into two characters)

25. Prismatic crystals: (0) absent, (1) rhombohedral, (2) spherical (S&P4, following arguments in Schmid, 1980 we have added the absent state (3) to S&P4. Spherical prismatic crystals defined in Retamales and Scharaschkin, 2015).

26. Leaf marginal sclerenchyma: (0) absent, (1) present (Observed in few Myrteae in Cardoso et al., 2009).

27. Foliar sclereids: (0) absent, (1) present (S&P15).

28. Bundle sheath: (0) parenchymatous, (1) sclerenchymatous (S&P9).

29. Secretory cavities: (0) schizogenous, (1) schizolysigenous, (2) absent (Retamales and Scharaschkin, 2015).

30. Oil deposits: (0) absent, (1) present. (Defined in S&P but not included in their phylogenetic analyses).

31. Shape of adaxial surface above vascular system: (0) flat/concave, (1) grooved, convex (S&P5).

32. Vascular system shape: (0) arc, (1) ellipsoid, (2) circular (S&P8, modified from S&P8 following Cardoso et al., 2009).

33. Lateral vascular system sheath extension: (0) absent, (1) present (S&P14).

34. Adaxial phloem partition: (0) absent, (1) weak, (2) strong (the adaxial phloem partition in some species may be well or poorly developed. In the latter case, this is scored as (1) present (S&P7)).

35. Adaxial-abaxial phloem confluence: (0) present, (1) absent (Cardoso et al., 2009 and Retamales and Scharaschkin, 2015).

36. Leaf adaxial phloem abundance: (0) scarce, (1) abundant (Cardoso et al., 2009 and Retamales and Scharaschkin, 2015).

37. Type of perforation plates: (0) only simple, (1) only scalariform, (2) mixed simple, reticulate and scalariform (LL4).

38. Vessel aggregation: (0) mostly solitary, (1) mostly grouped (scored based on Ingle and Dadswell, 1953 and Ragonese, 1976).

39. Presence of helical wall thickenings: (0) absent, (1) present (Schmid, 1980, 1984).

40. Presence of fibre-tracheids: (0) present, (1) absent (W3, J&B5, presence of fibre-tracheids usually interpreted as the plesiomorphic condition (Wilson et al. 2001)).

41. Wood rays: (0) weakly heterogeneous, (1) strongly heterogeneous (W6, J&B10, rays in Myrtaceae are never homogeneous. Distinctions between heterogeneity taken from Ingle and Dadswell (1953)).

42. Silica in wood rays: (0) absent, (1) present (Ragonese, 1976).

43. Inflorescence branching: (0) much branched, (1) slightly reduced, (2) reduced to triads or monads (W17, J&B27, 28).

44. Dichasial inflorescence: (0) common, (1) absent or very rare (L25).

45. Bracteate shoot inflorescence: (0) common, (1) absent or very rare (L26).

46. Pubescence on the peduncles: (0) dense, (1) sparse or variable, (2) lacking or essentially lacking (L17).

47. Solitary peduncles: (0) peduncles essentially always solitary, (1) some peduncles not solitary (L27).

48. Number of sepals and petals: (0) five, (1) four (W41, J&B32).

49. Pubescence on sepals: (0) dense, (1) sparse or variable, (2) lacking or essentially lacking (L18).

50. Pubescence on petals: (0) dense, (1) sparse or lacking (L29).

51. Type of hairs on petals and sepals: (0) simple, (1) dibrachiate (character states gathered from Landrum, 1981a, Landrum and Kawasaki, 1997 and Wilson, 2011).

52. Stamen position: (0) erect, (1) semi-curved, (2) strongly curved (W23, J&B37, modified according to Vasconcelos et al., 2005 for Myrteae).

53*. Approximate number of stamens per flower: (0) more than 100, (1) fewer than 30 (Landrum, 1981a, Landrum and Kawasaki, 1997 and Wilson, 2011).

54. Pollen colpus morphology: (0) brevicolpate, (1) parasyncolpate, (2) syncolpate, (3) dicolpate (W26, J&B39, 40, adapted following Thornhill and Crisp, 2012).

55. Number of ovules per locule: (0) Fewer than six, (1) between seven and 20, (2) more than 20 (W43, J&B59, 60).

56. Number of locules per ovary: (0) two, (1) three, (2) four, (3) more than four (LL2).

57. Mean number of ovules per ovary: (0) fewer than 70, (1) more than 120 (Lucas et al., 2007).

58. Type of placentation: (0) ovules mostly arising at a single point on septum, (1) ovules in one or more series along a non-peltate placenta, (2) ovules in one or more series around a protruding placenta (LL3).

59. Number of carpels: (0) two, (1) three, (2) four (Landrum, 1988, Landrum and Kawasaki, 1997 and Wilson, 2011).

60. Ovulodes: (0) absent, (1) present (W42, J&B55).

61. Nature of pericarp in fruit: (0) dry, (1) fleshy (W35, J&B58).

62. Type of embryo: (0) hypocotyl wrapped around well-developed, crumpled cotyledons, (1) hypocotyl much reduced, cotyledons swollen, homogeneous or fused, (2) hypocotyl swollen, usually wider than the cotyledons, sometimes spiralled, (3) cotyledons inconspicuous, hypocotyl linear or C-shaped, (4) short hypocotyl enclosed by entire, plano-convex cotyledons, (5) short hypocotyl enclosed by lobed and thickened cotyledons (LL1).

63. Number of seeds relative to the number of ovules per locule: (0) seeds numerous and potentially equal to ovule number, (1) seeds few per fruit, (2) only one seed developed per fruit (W43, J&B59,60, Wilson et al., 2001 combined characters number 59 and 60 from Johnson and Briggs, 1984).

64. Vascularization of the ovary: (0) axile vascularization only, (1) mixed (axile and transeptal) vascularization present, (2) transeptal vascular traces present (W30, J&B45).

Appendix 2 continuation.

65. Number of receptacular vascular bundles: (0) indefinite number, (1) definite number (eight), (2) definite number (five) (P3, character state (2) observed and included from this investigation).

66*. Nectariferous region: (0) inconspicuous, (1) conspicuous.

67*. Secretory cavities in petals: (0) present, (1) absent.

68. Secretory cavities in anthers: (0) present, (1) absent (Schmid, 1972, 1980).

69. Number of epidermal layers in sepals: (0) one, (1) two or more (Schmid, 1972, 1980).

70. Development of connective tissue: (0) poorly developed, (1) well developed (Schmid, 1972, 1980 and mentioned in Wilson, 2011).

71. Crystals in anthers: (0) present, (1) absent (Schmid, 1972, 1980).

72. Number of vascular bundles in sepals: (0) four or less, (1) more than four (Schmid, 1972, 1980).

73. Number of vascular bundles in petals: (0) four or less, (1) more than four (Schmid, 1972, 1980).

74. Origin of placenta: (0) cauline, (1) carpellate (P2).

75. Tannin content in mesophyll of leaves: (0) abundant, (1) scarce, (2) absent (Schmid, 1972, 1980. Character states constructed for this investigation).

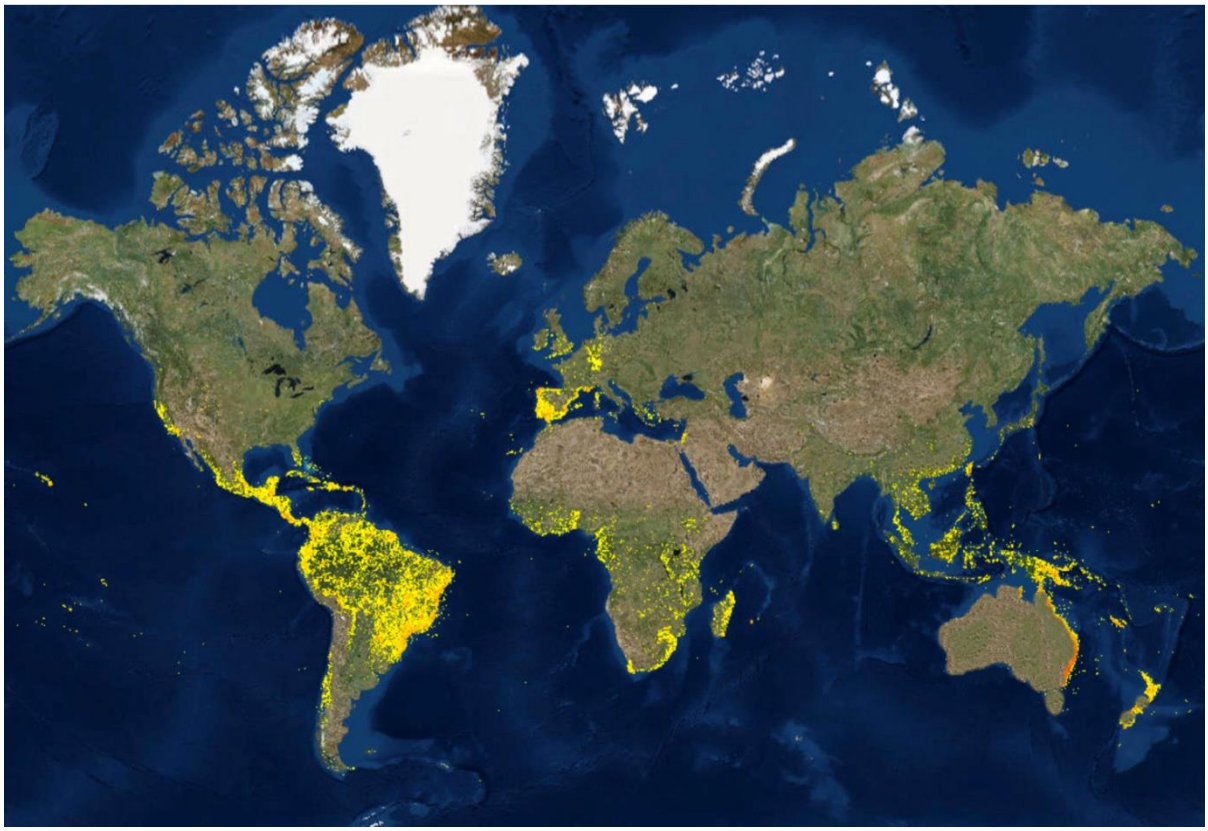
76. Polyphenol content in cuticle of leaves: (0) abundant, (1) scarce, (2) absent (Schmid, 1972, 1980. Character states constructed for this investigation).

77. Tannin content in mesophyll of petals: (0) abundant, (1) scarce (Schmid, 1972, 1980. Character states constructed for this investigation).

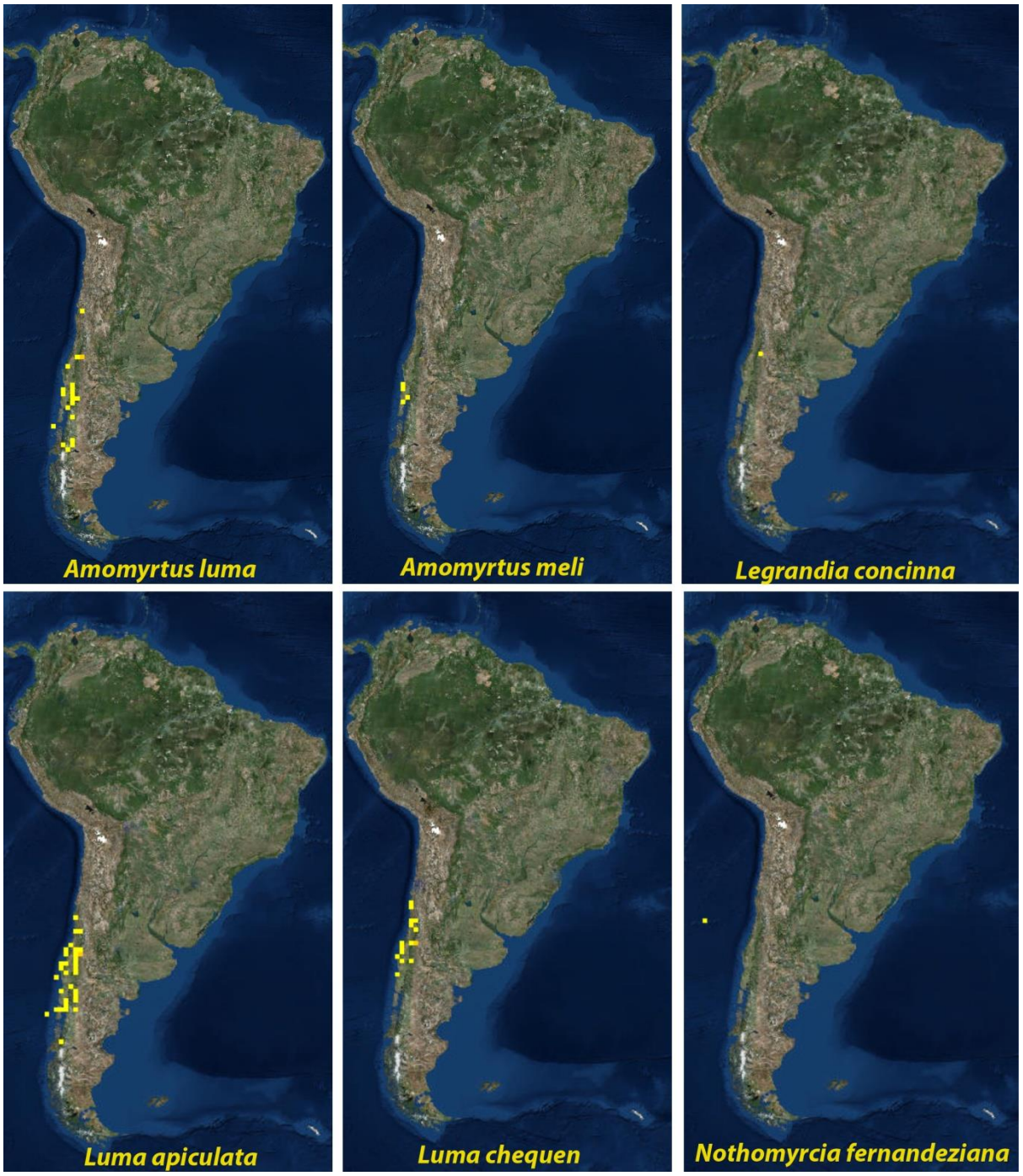
78. Mucilage content in epidermis: (0) abundant, (1) scarce, (2) absent (Schmid, 1972, 1980. Character states constructed for this investigation).

79. Tannin content in phloem of flowers: (0) abundant, (1) scarce (Schmid, 1972, 1980. Character states constructed for this investigation).

Appendix 3. Distribution maps for taxa used in this investigation. Geographic distribution of individual species shown only for Chilean Myrteae endemic to the temperate forests of Chile and Argentina (including the Juan Fernandez Islands). Yellow dots indicate geographic distribution by voucher specimens collected in herbaria. Maps obtained from GBIF (Global Biodiversity Information Facility - www.gbif.org).



Geographic distribution of the tribe Myrteae.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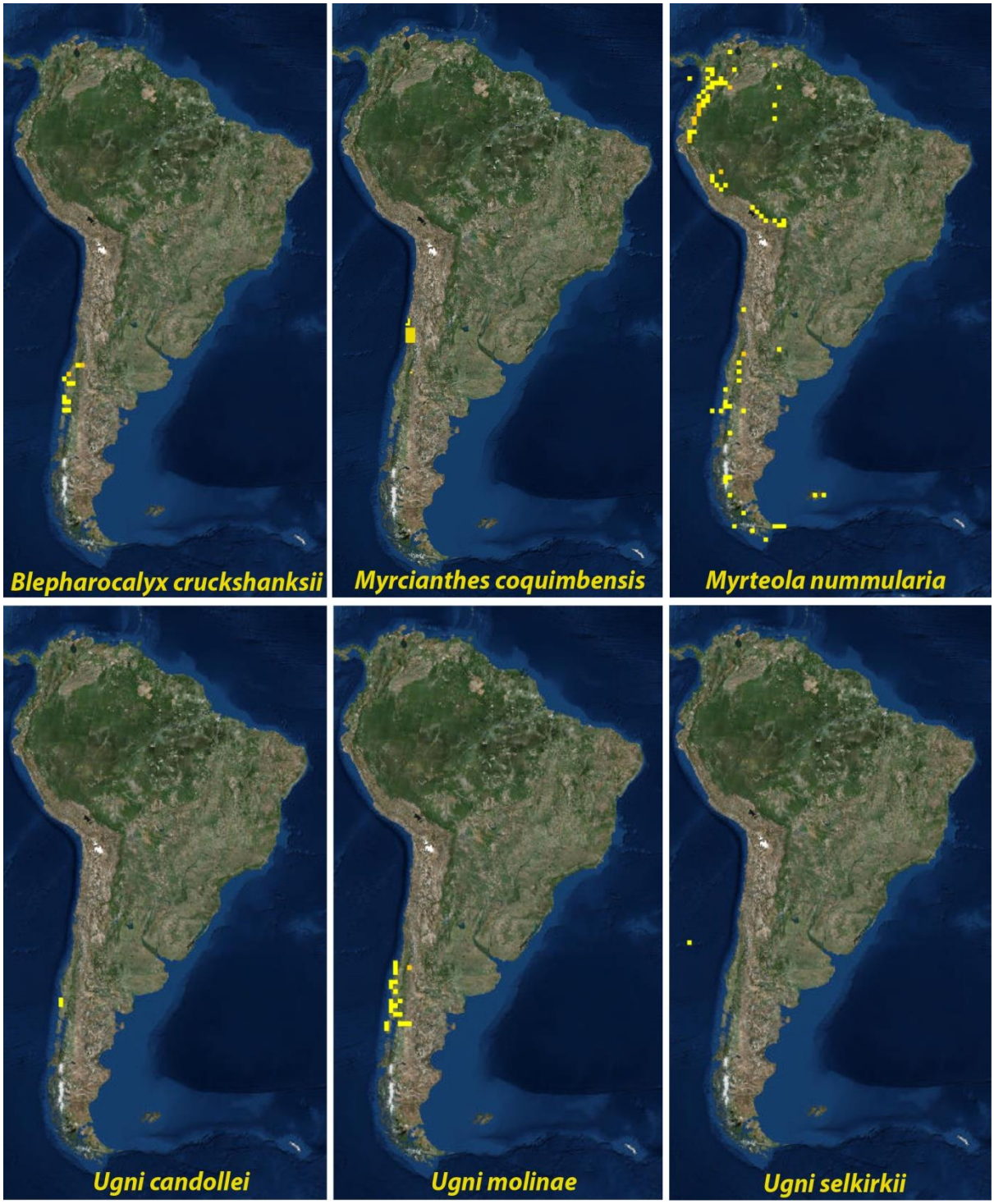
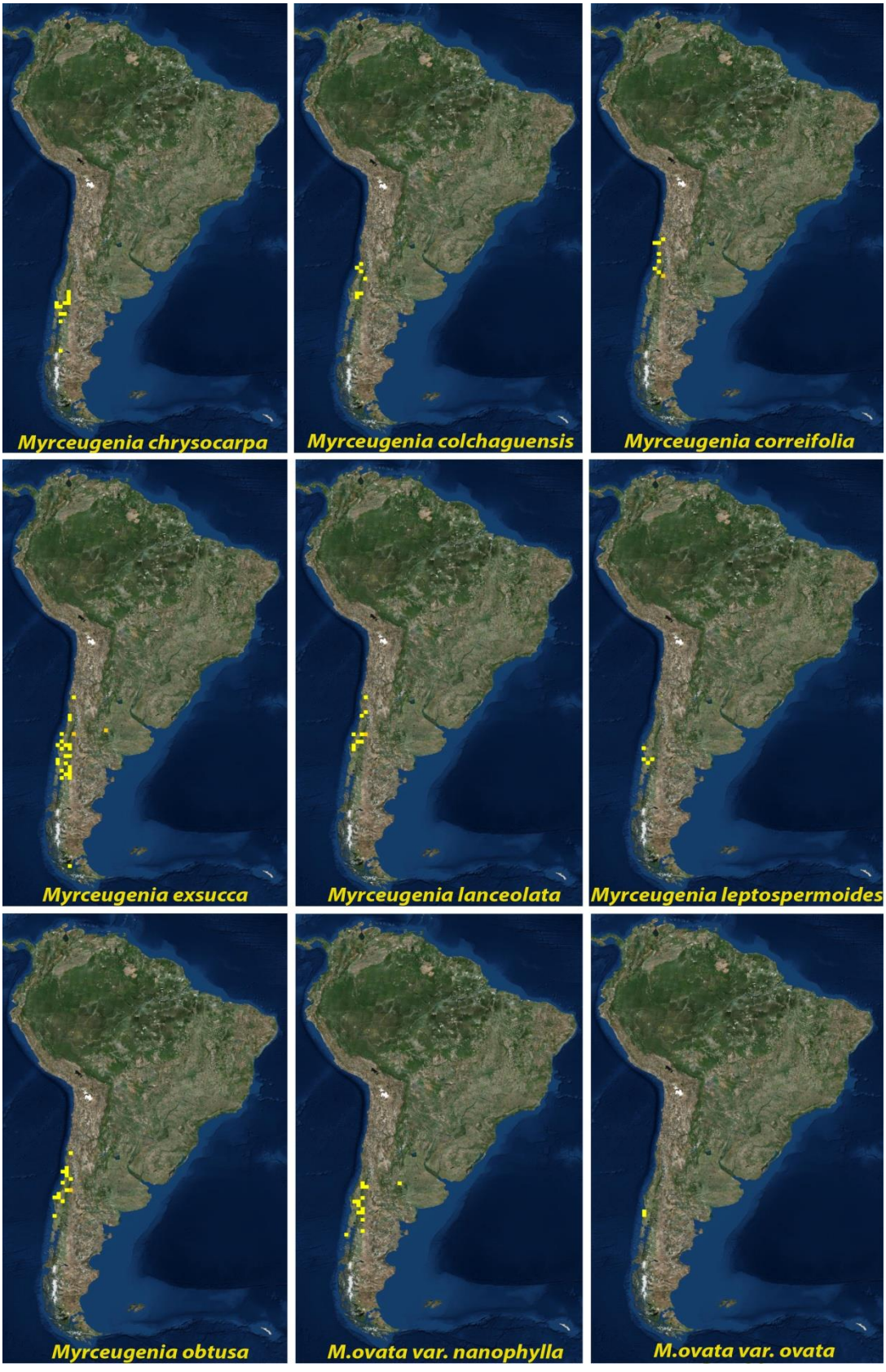
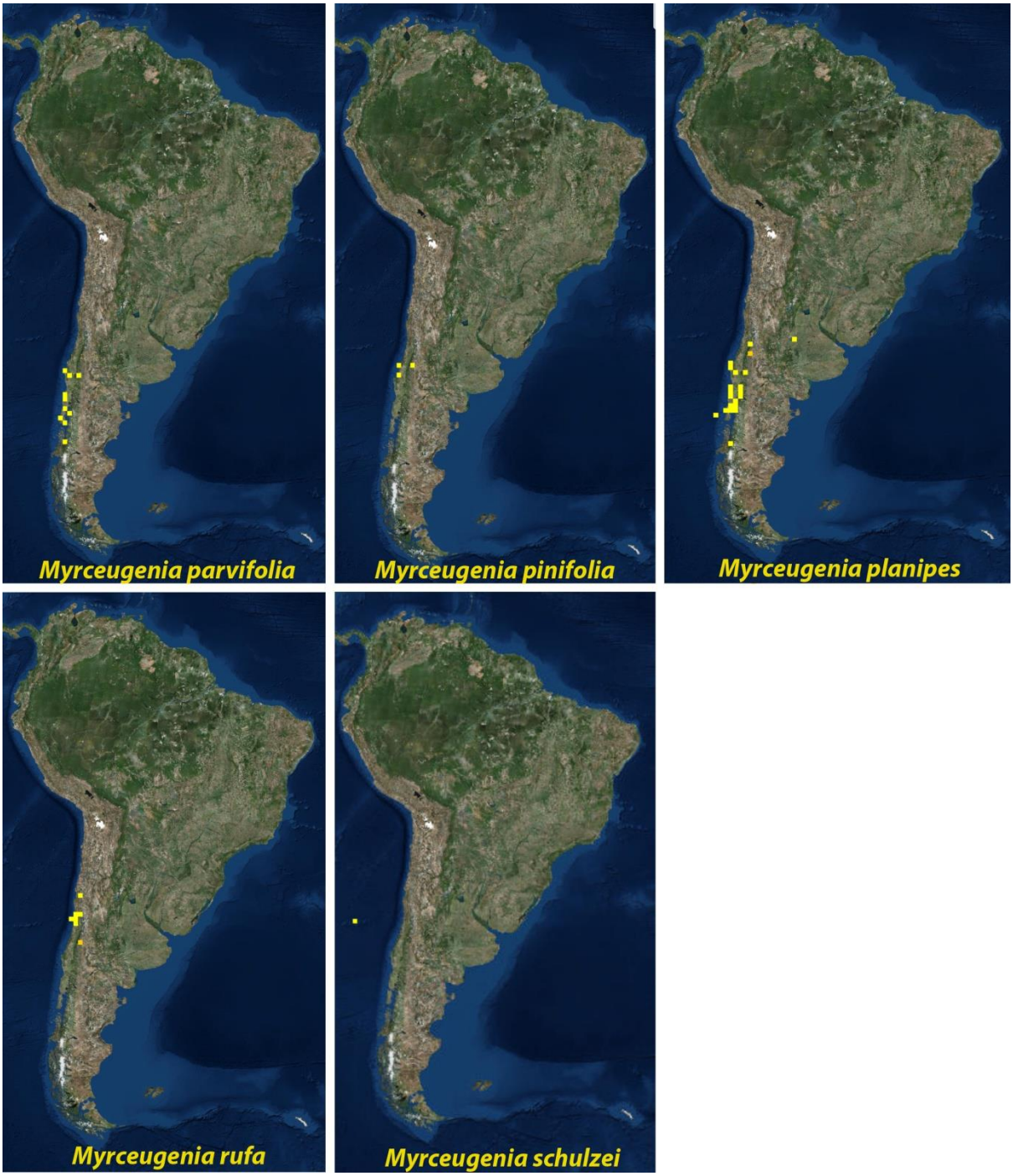
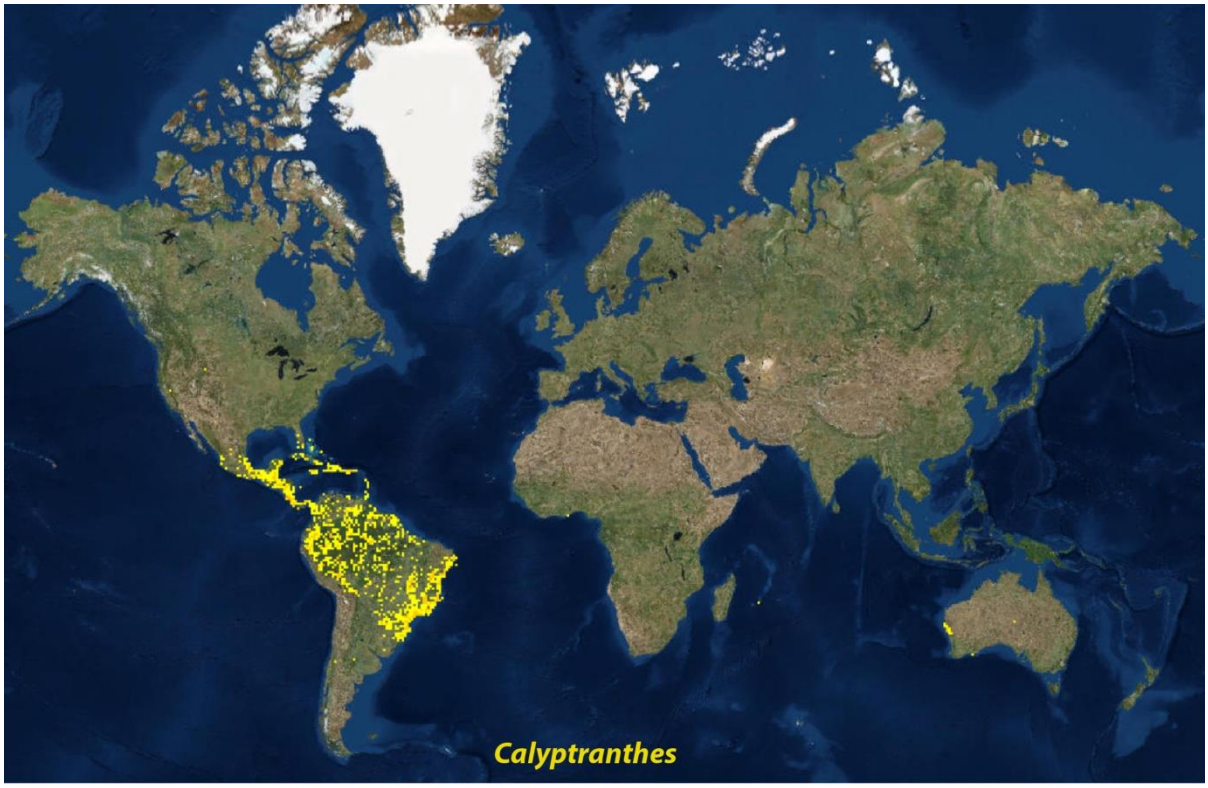
Amomyrtus meli
Legrandia concinna
Luma apiculata
Luma chequen
Nothomyrcia fernandeziana

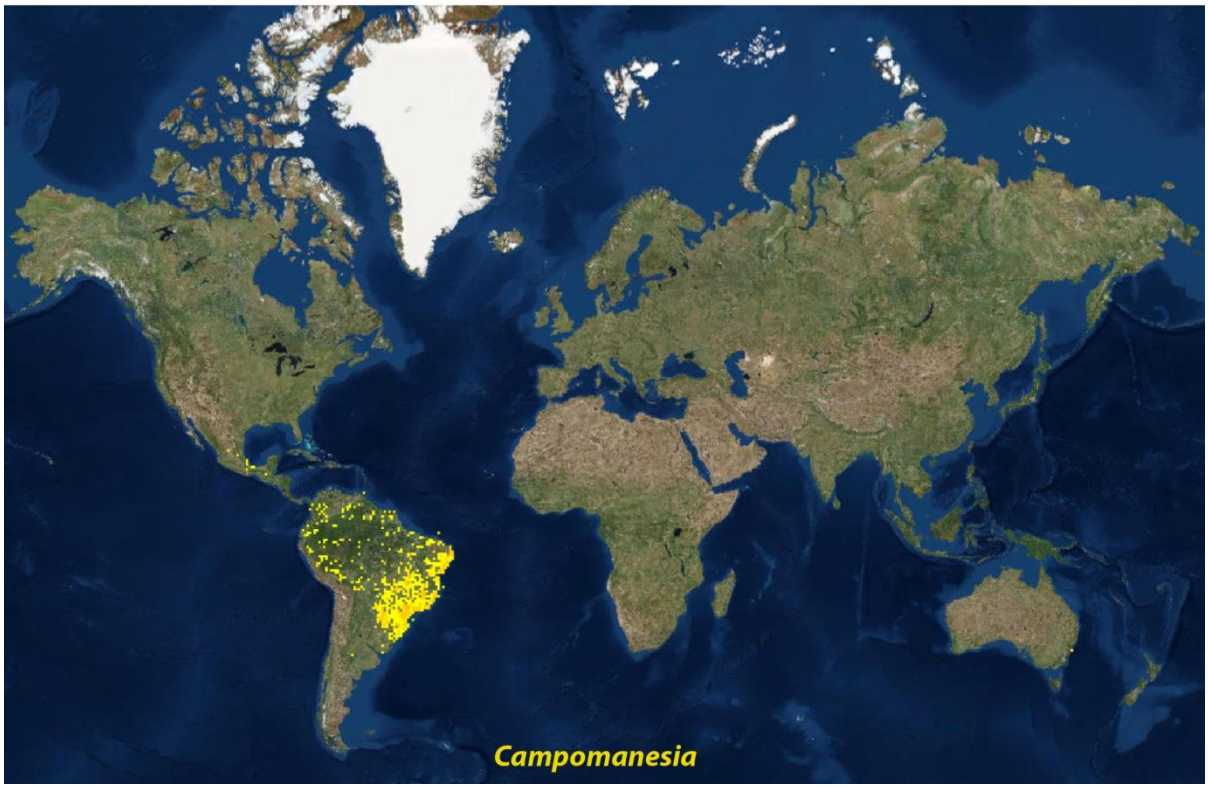

Blepharocalyx cruckshanksii
Myrcianthes coquimbensis
Myrteola nummularia
Ugni candollei
Ugni molinae
Ugni selkirkii

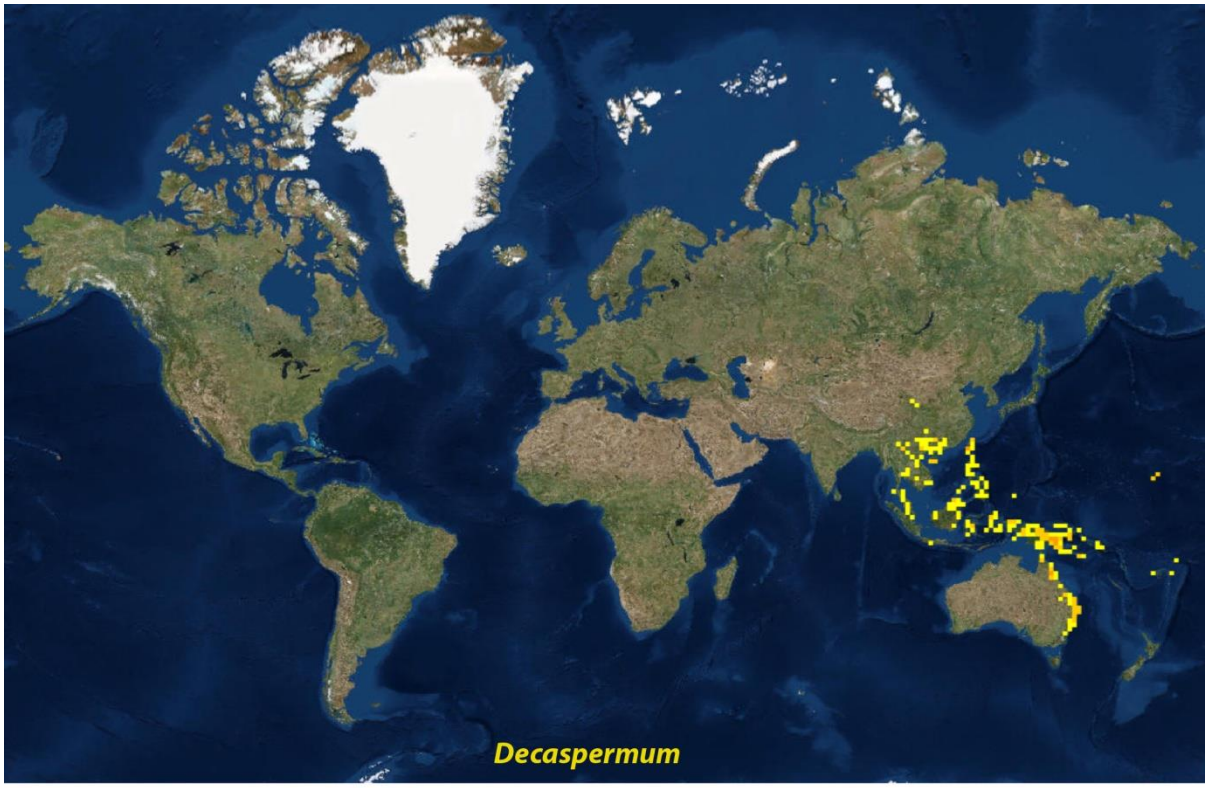

Myrceugenia chrysocarpa
Myrceugenia colchaguensis
Myrceugenia correifolia
Myrceugenia exsucca
Myrceugenia lanceolata
Myrceugenia leptospermoides
Myrceugenia obtusa
M.ovata var. nanophylla
M.ovata var. ovata

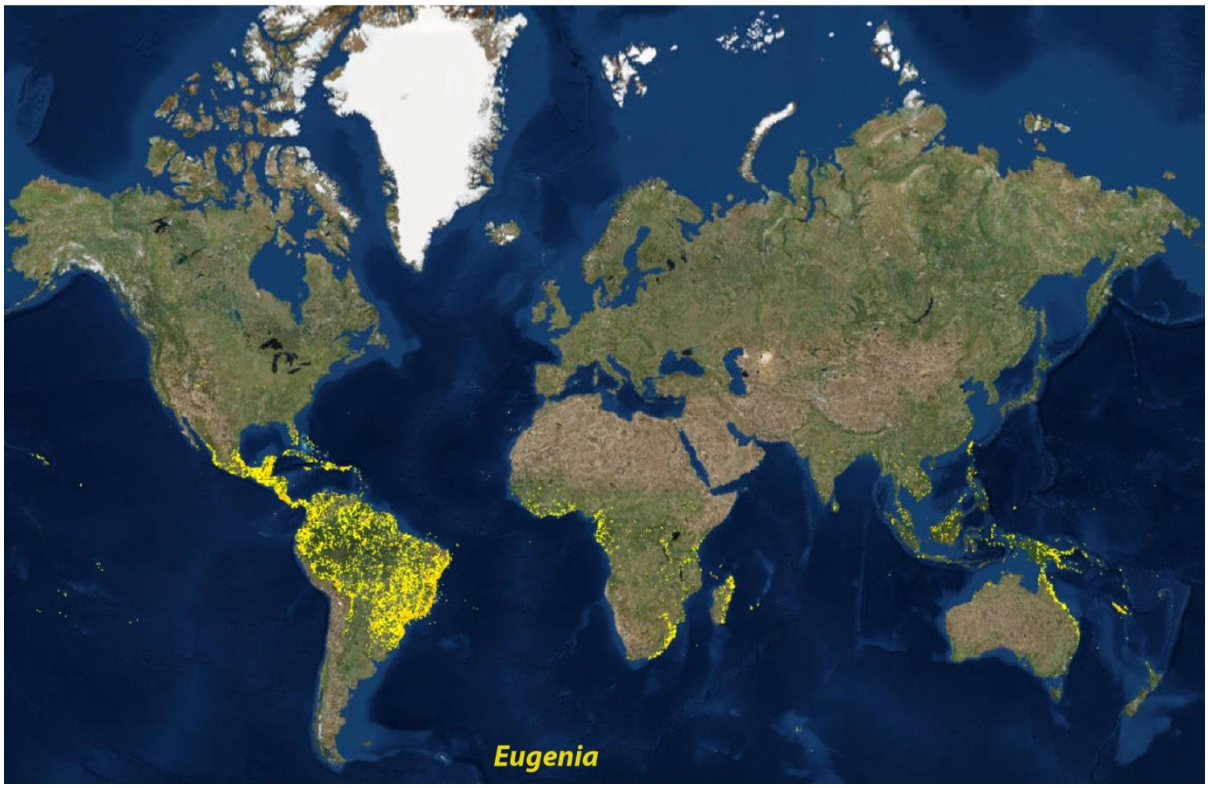

Myrceugenia parvifolia
Myrceugenia pinifolia
Myrceugenia planipes
Myrceugenia rufa
Myrceugenia schulzei

Geographic distribution of *Myrtus*.


Calyptranthes


Campomanesia


Decaspermum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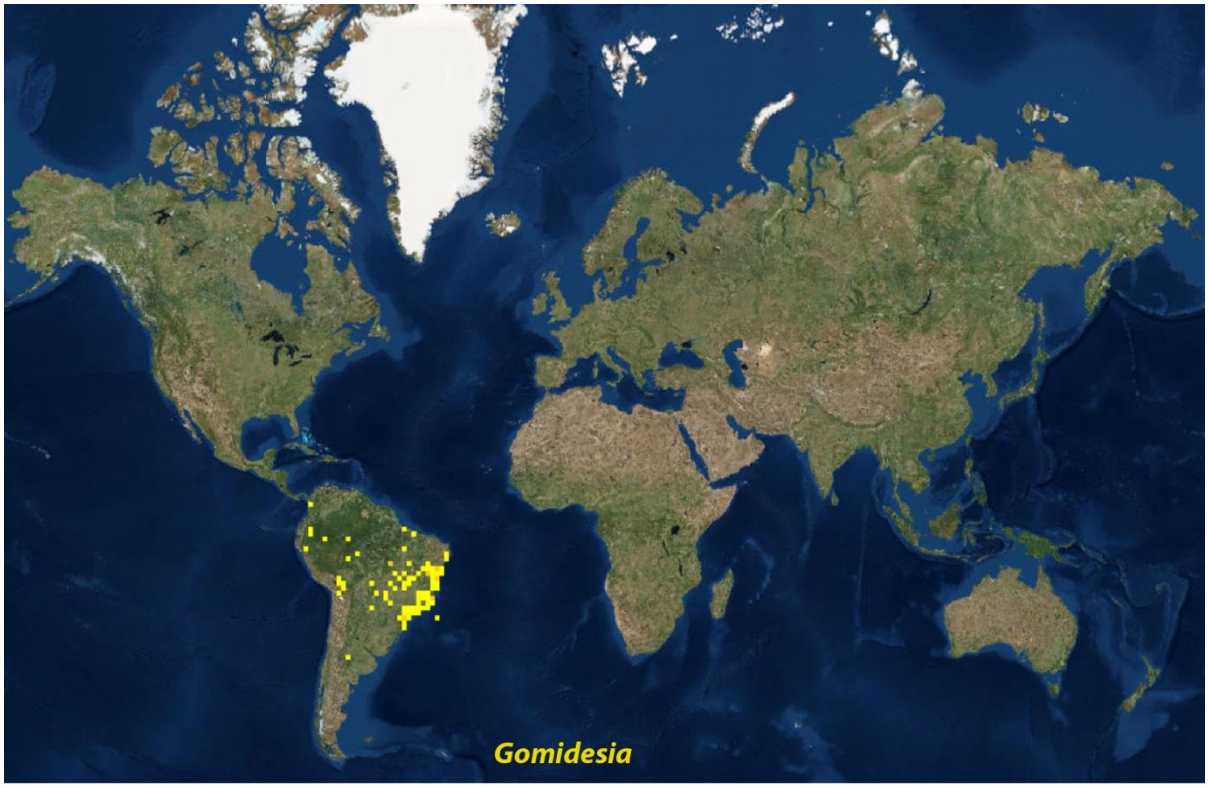
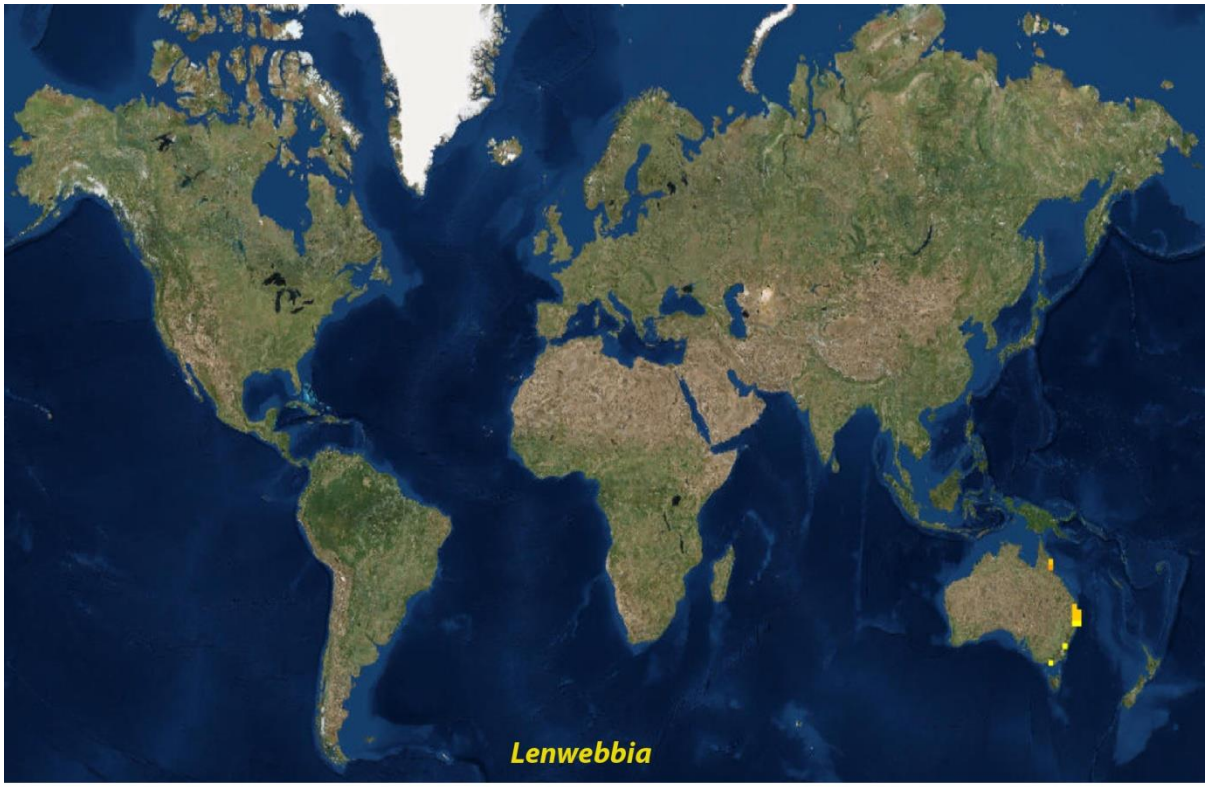
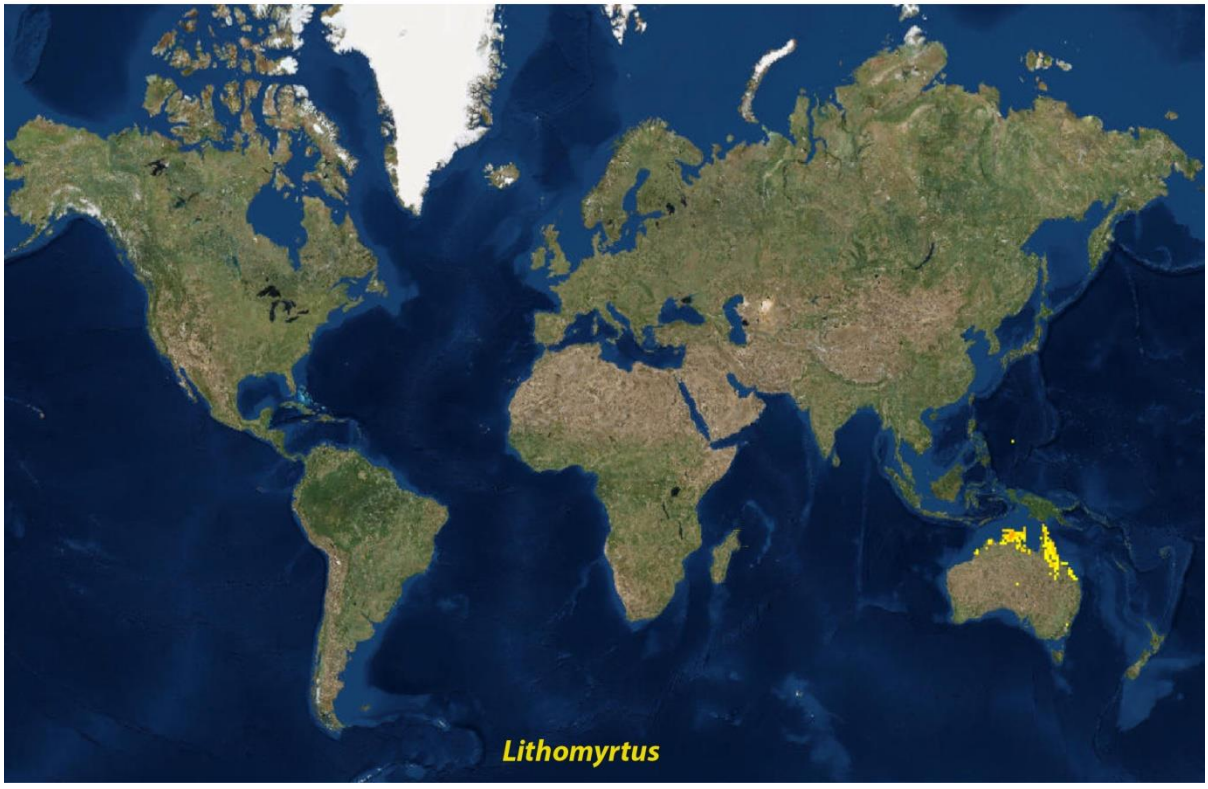
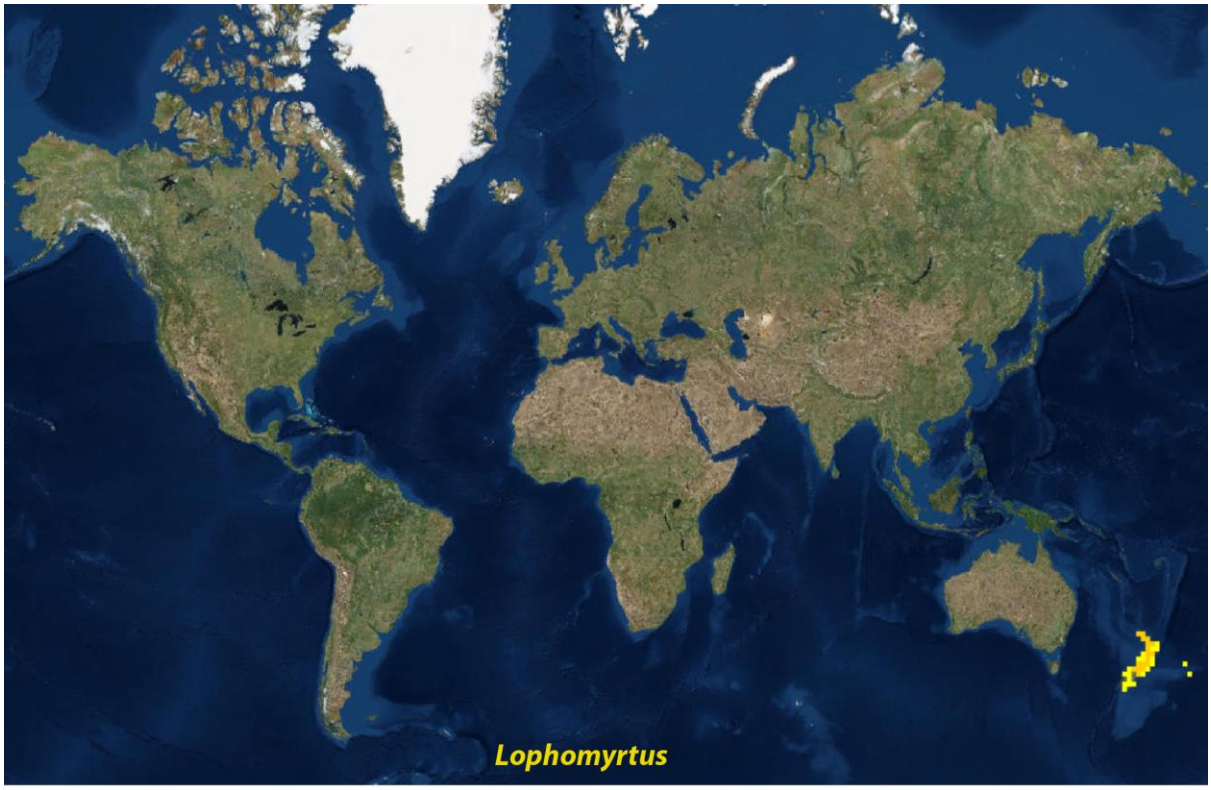
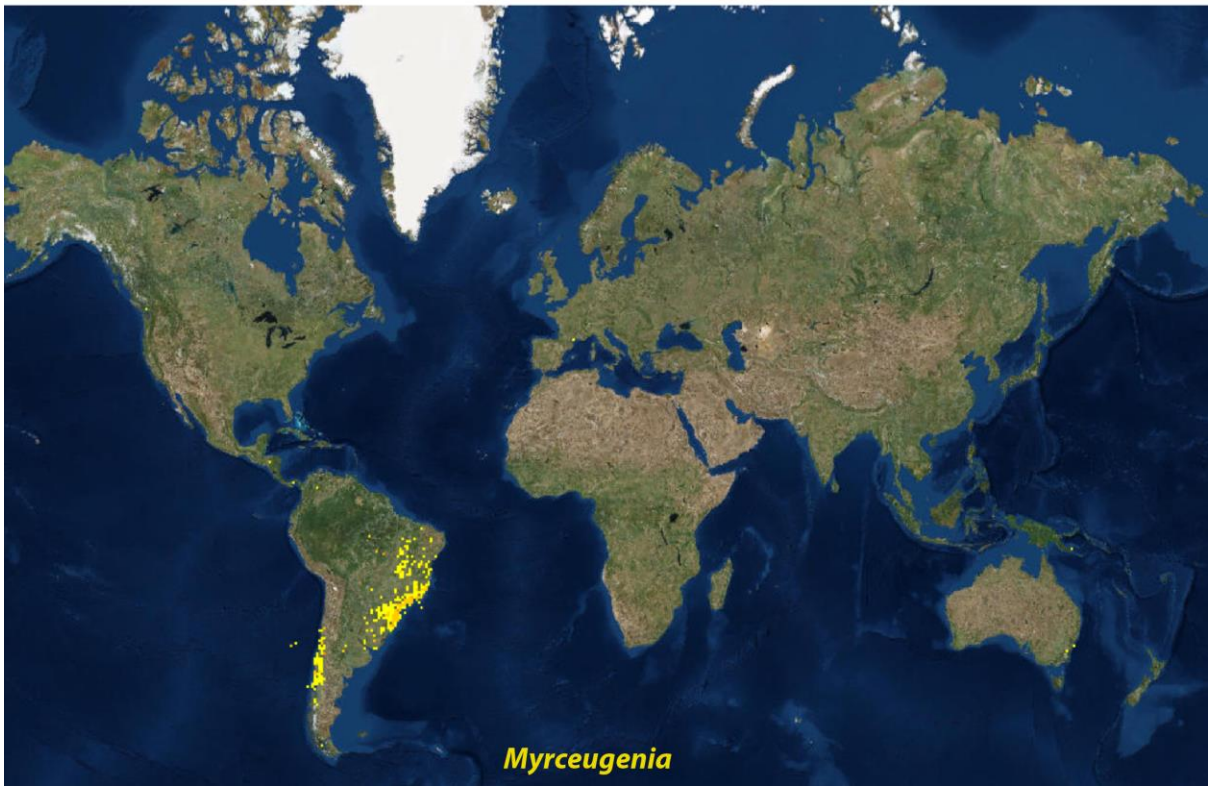

Gomidesia

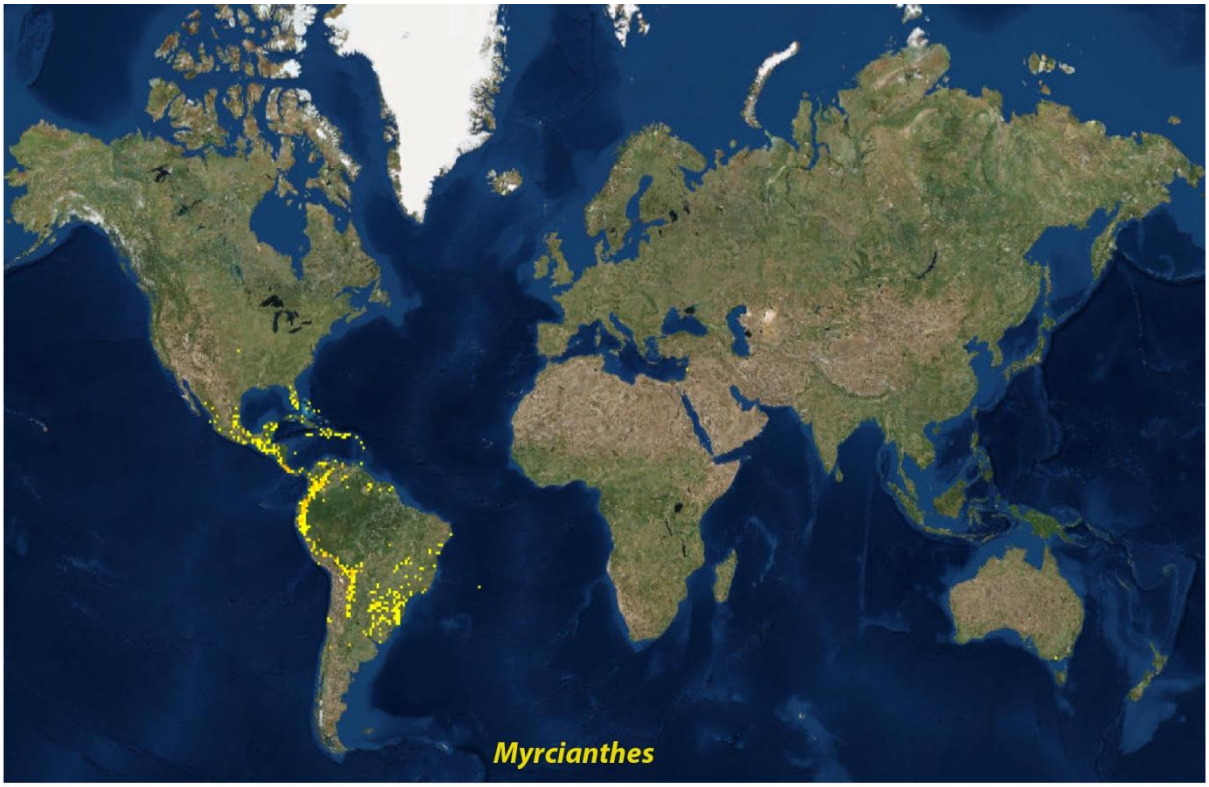

Gossia

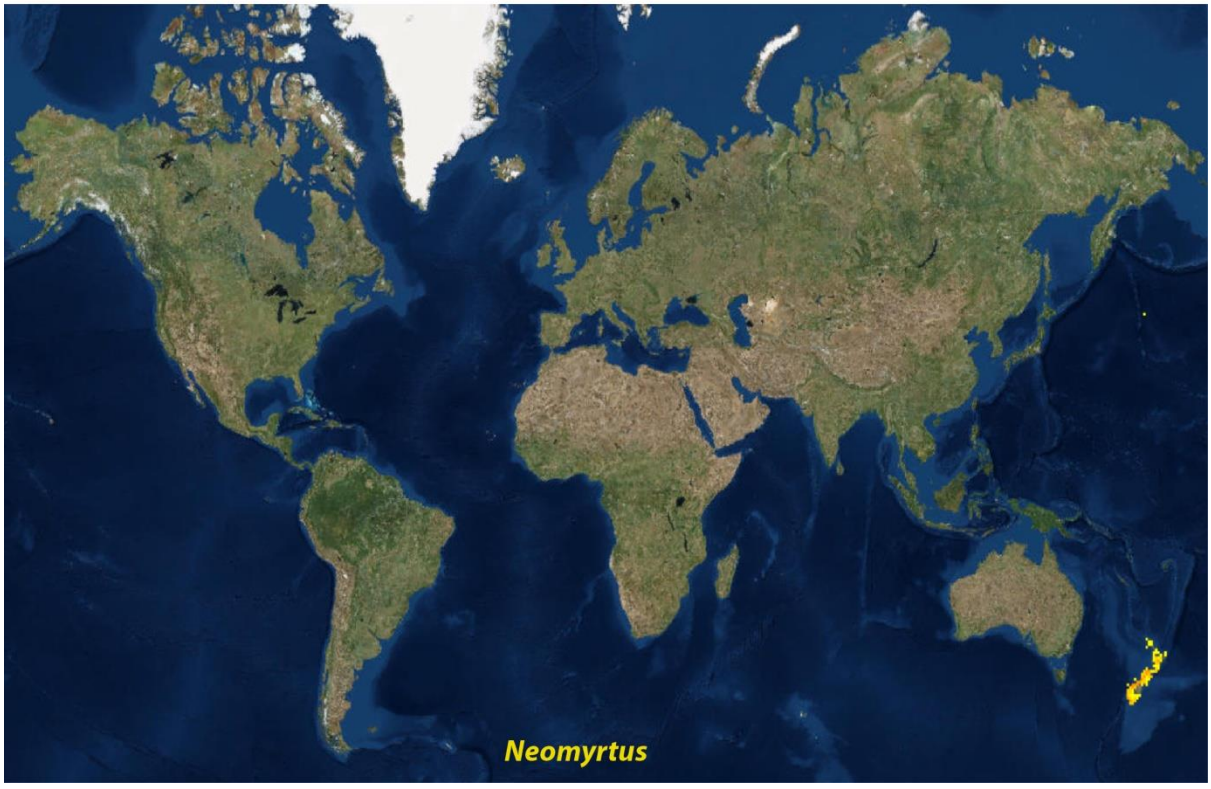

Lenwebbia

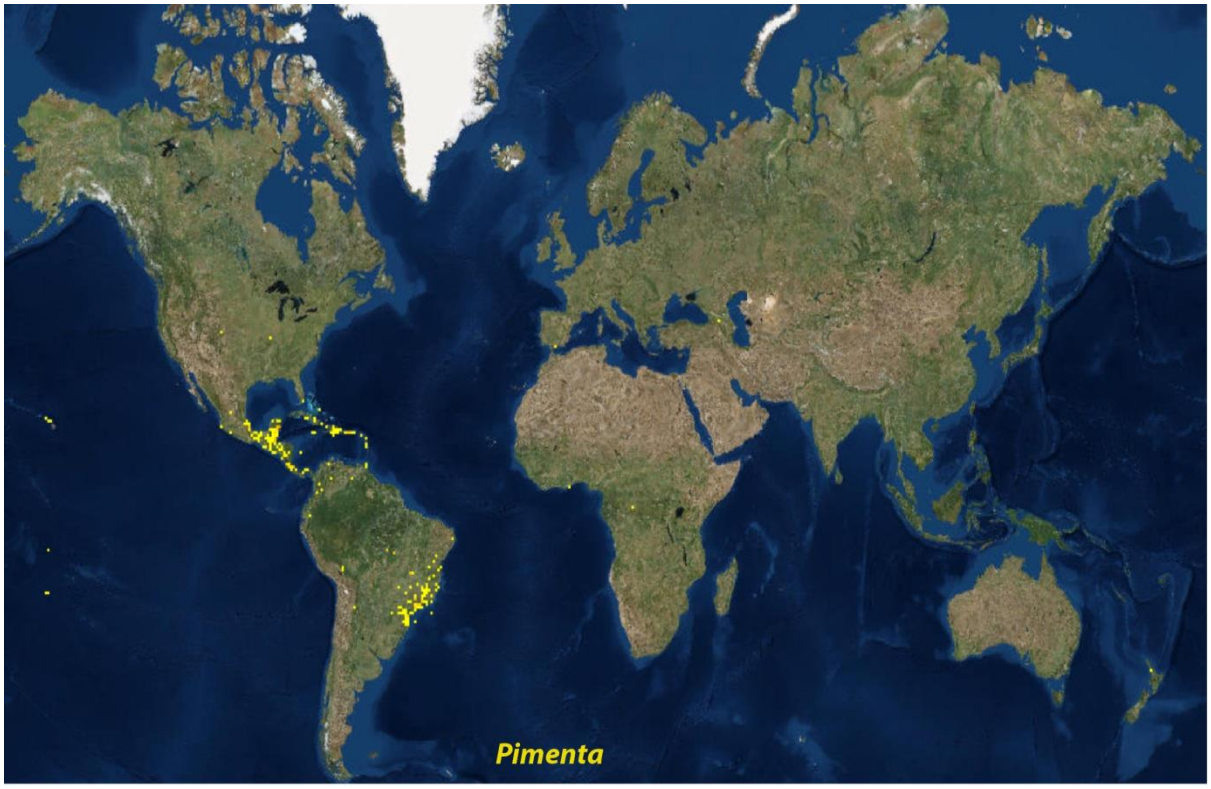

Lithomyrtus

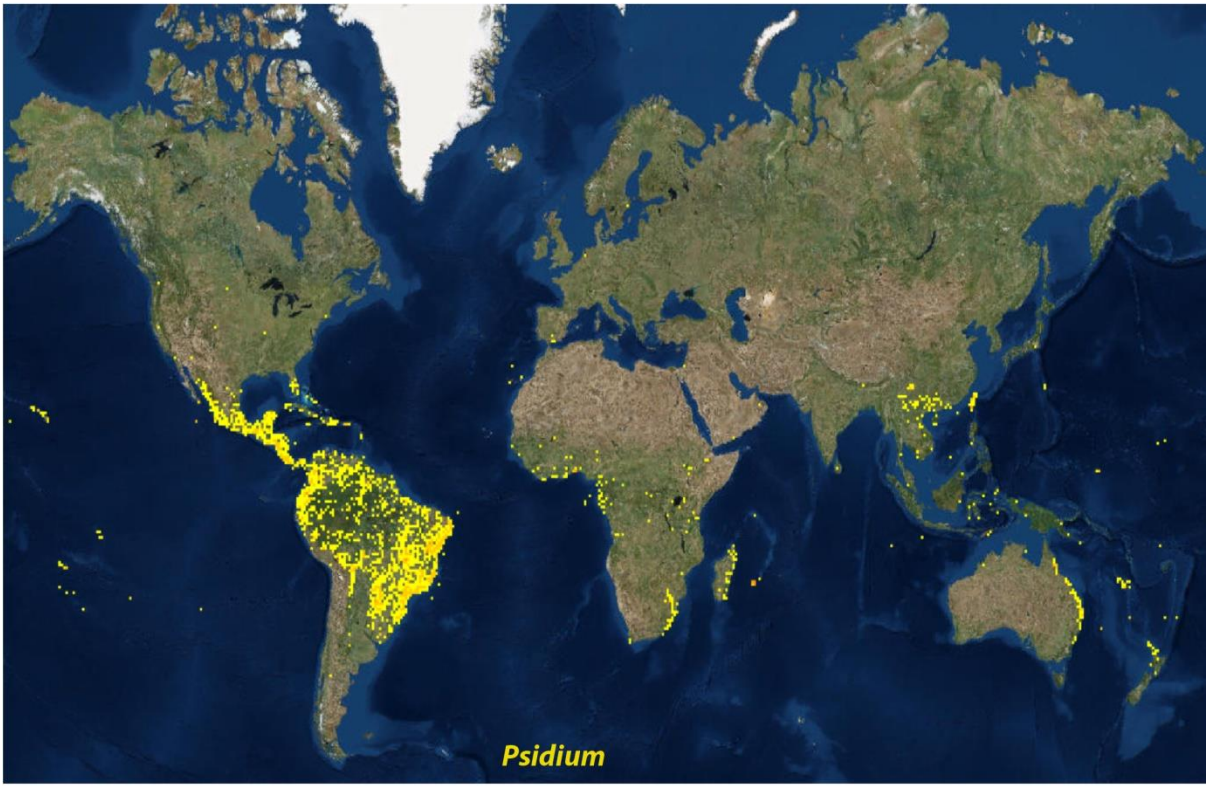

Lophomyrtus


Myrceugenia

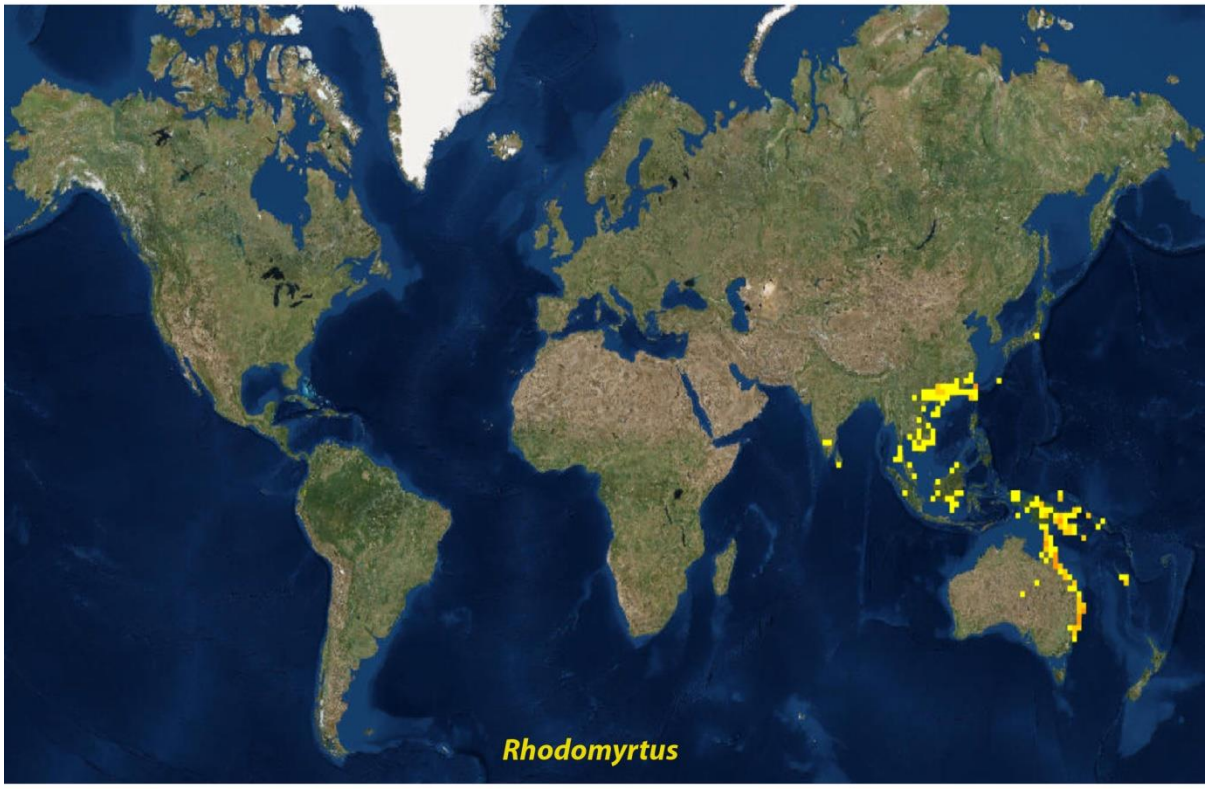

Myrcianthes


Neomyrtus


Pimenta


Psidium


Rhodamnia


Rhodomyrtus

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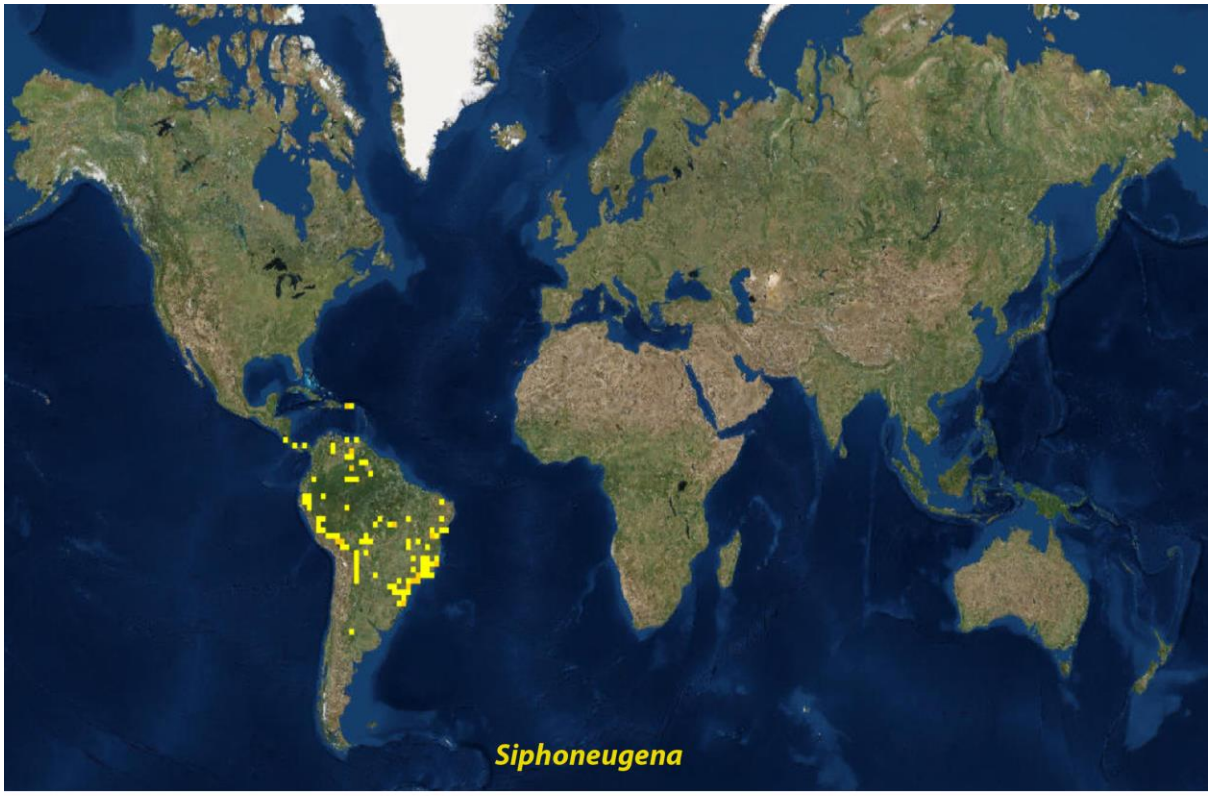
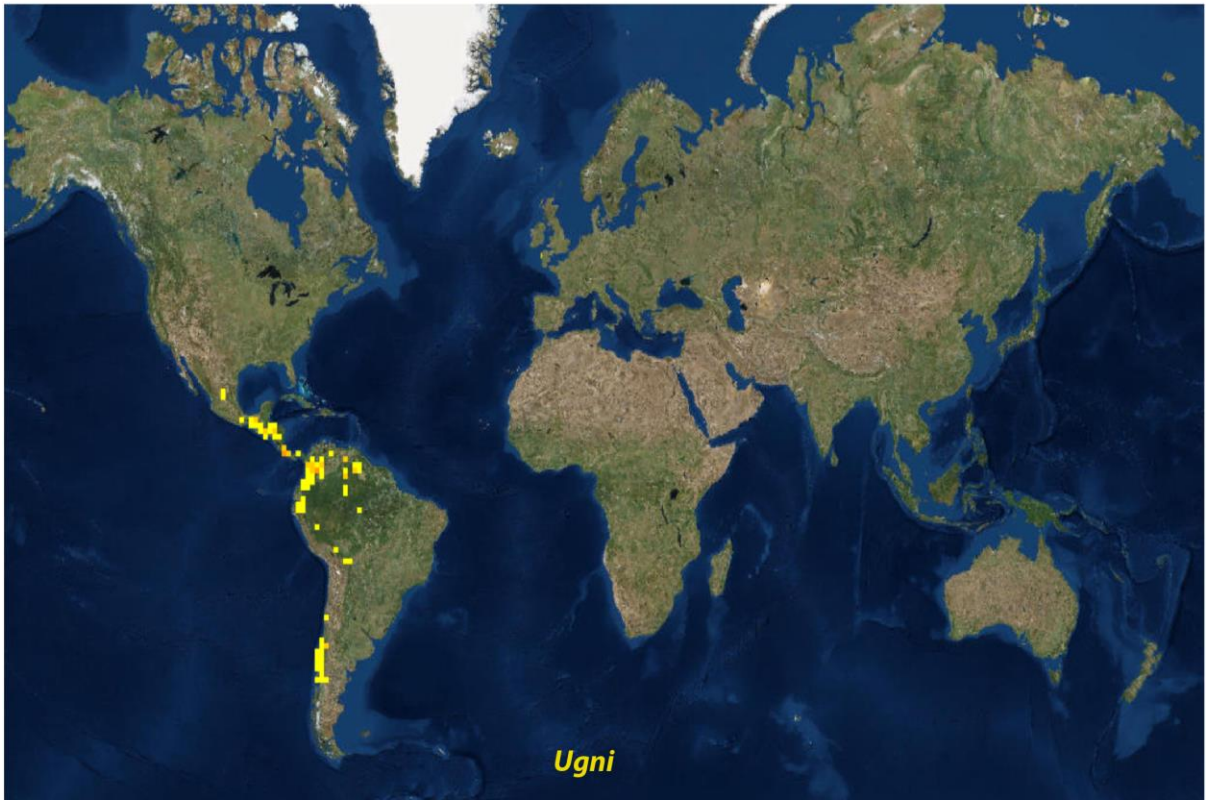

Siphoneugena


Ugni

Appendix 4. Description of the staining protocol (Chapter 3) and troubleshooting.

Chemicals (and vendors/suppliers) used in this study:

Xylene (Ajax Finechem Pty, Taren Point, New South Wales, Australia) CAS: 1330-20-7

Ethanol (Chem-Supply Pty, Gillman, South Australia, Australia) CAS: 64-17-5

Ruthenium red powder (Sigma-Aldrich Co., Saint Louis, Missouri, USA). CAS: 11103-72-3

Toluidine blue powder (TBO) (Amresco Co., Solon, Ohio, USA). CAS: 92-31-9

DPX (Sigma-Aldrich Co., Saint Louis, Missouri, USA). CAS: 14208-10-7

Distilled water

Equipment

Safety cabinet, microtome, drying oven, microscope, glass staining dishes with glass lids, slide racks, microscope slides, coverslips (24x24mm) and long coverslips (24x50mm), trays for drying slides, NitriSolve flock-lined gloves are recommended when handling xylene, and nitrile gloves for all other reagents, safety goggles, paper towels, dropper bottle, forceps, spoon, spatula.

Preparation of staining solutions

Ruthenium red (Jensen, 1962)

1. Dissolve 0.3 g of Ruthenium red powder in 100 ml of distilled water.

2. Stir for 15-20 minutes until dissolved, without heat, until completely dissolved.

3. Filter after stirring

4. Store in Schott bottle, refrigerated to prevent growth of microorganisms

Freshly prepared solution should look black in colour.

Toluidine blue (O'Brien et al., 1964)

1. Dissolve 0.1 g of Toluidine blue in 100 ml of distilled water.

2. Stir gently

3. Filter

4. Store in Schott bottle, refrigerated to prevent growth of microorganisms

Histochemical staining procedure protocol

1. Place 20 staining dishes in a fumehood. Fill the dishes with the following solutions: 2x 50% ethanol, 2x 70% ethanol, 2x 90% ethanol, 2x ethanol 100%, 4x xylene, 1x xylene:ethanol (1:3), 5x distilled water, 1x aqueous solution of 0.05% (w/v) Ruthenium red and 1x aqueous solution of 0.1% (w/v) Toluidine blue.

2. Place slides with paraffin sections in slide racks and transfer to different solutions and stains using the sequence and duration outlined below:

Step	Solution	Purpose	Duration (estimated intervals)
1-2	Xylene	Deparaffinisation	15 min (two changes)
3	Xylene:Ethanol	Hydration	10 min
4	100% Ethanol	Hydration	5 min
5	90% Ethanol	Hydration	5 min
6	70% Ethanol	Hydration	5 min
7	50% Ethanol	Hydration	5 min
8	Distilled water	Hydration	5 min
9	Ruthenium red	Staining	Variable
10	Distilled water	Removal of excess stain	30 sec
11	Distilled water	Removal of excess stain	30 sec
12	Toluidine blue	Counter staining	Variable
13	Distilled water	Removal of excess stain	30 sec
14	Distilled water	Removal of excess stain	30 sec

Step	Solution	Purpose	Duration (estimated intervals)
15	50% Ethanol	Dehydration	5 min
16	70% Ethanol	Dehydration	5 min
17	90% Ethanol	Dehydration	5 min
18	100% Ethanol	Dehydration	5 min
19-20	Xylene	Drying	10 min (two changes)

3. Check staining result under a microscope after step 14 before proceeding with dehydration. Adjust staining time if needed.

4. Mount and coverslip with DPX (Entellan®)

5. Lay coverslips out on a sheet of blotting paper and wipe clean to remove dust. Place an elongated drop of mountant (DPX) across the middle of each coverslip. Remove a slide from the xylene (Step 20 above), drain off the excess liquid and lay it, section side down, over the coverslip. Repeat for each slide, making sure that air bubbles are not trapped beneath the coverslip. If bubbles are present, press them gently towards the edge of the coverslip with a mounted needle. Wipe around the slide with a tissue to remove excess xylene or mountant. Dry the slides on a flat surface in the fume hood until the mountant has hardened sufficiently to allow handling.

Appendix 5. List of publications produced during the course of the PhD.

Retamales, H. and T. Scharaschkin. 2015. Comparative leaf anatomy and micromorphology of the Chilean Myrtaceae: Taxonomic and ecological implications. *Flora* 217: 138-154

Retamales, H. and T. Scharaschkin. 2014. A staining protocol for identifying secondary compounds in Myrtaceae. *Applications in Plant Sciences* 2(10): 1-8.

Retamales, H., A. Cabello, M.T. Serra and T. Scharaschkin. 2014. Anatomical studies of the flower, fruit and seeds of *Myrceugenia rufa* (Myrtaceae). *Boletin del Museo Nacional de Historia Natural* 63: 89-100.

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Retamales, H., R. Scherson and T. Scharaschkin. Phylogenetic placement of the Chilean Myrteae (Myrtaceae) using molecular and morphological evidence. *Plant Systematics and Evolution*.

Retamales, H., R. Scherson and T. Scharaschkin. Character evolution in Myrteae (Myrtaceae). *International Journal of Plant Sciences.*

Applications in Plant Sciences 2014 2(10): 1400063

Protocol Note

A staining protocol for identifying secondary compounds in Myrtaceae[1]

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- *Premise of the study:* Here we propose a staining protocol using toluidine blue (TBO) and ruthenium red to reliably identify secondary compounds in the leaves of some species of Myrtaceae.
- *Methods and Results:* Leaves of 10 species representing 10 different genera of Myrtaceae were processed and stained using five different combinations of ruthenium red and TBO. Optimal staining conditions were determined as 1 min of ruthenium red (0.05% aqueous) and 45 s of TBO (0.1% aqueous). Secondary compounds clearly identified under this treatment include mucilage in the mesophyll, polyphenols in the cuticle, lignin in fibers and xylem, tannins and carboxylated polysaccharides in the epidermis, and pectic substances in the primary cell walls.
- *Conclusions:* Potential applications of this protocol include systematic, phytochemical, and ecological investigations in Myrtaceae. It might be applicable to other plant families rich in secondary compounds and could be used as a preliminary screening method for extraction of these elements.

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Comparative leaf anatomy and micromorphology of the Chilean Myrtaceae: Taxonomic and ecological implications

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ABSTRACT

The family Myrtaceae in Chile comprises 26 species in 10 genera. The species occur in a diverse range of environments including humid temperate forests, swamps, riparian habitats and coastal xeromorphic shrublands. Most of these species are either endemic to Chile or endemic to the humid temperate forests of Chile and Argentina. Although many taxa have very restricted distributions and are of conservation concern, little is known about their biology and vegetative anatomy. In this investigation, we describe and compare the leaf anatomy and micromorphology of all Chilean Myrtaceae using standard protocols for light and scanning electron microscopy. Leaf characters described here are related to epidermis, cuticle, papillae, stomata, hairs, mesophyll, crystals, secretory cavities and vascular system. Nearly all the species have a typical mesophytic leaf anatomy, but some species possess xerophytic characters such as double epidermis, hypodermis, pubescent leaves, thick adaxial epidermis and straight epidermal anticlinal walls, which correlate with the ecological distribution of the species. This is the first report on leaf anatomy and micromorphology in most of these species. We identified several leaf characters with potential taxonomic and ecological significance. Some combinations of leaf characters can reliably delimitate genera, while others are unique to some species. An identification key using micromorphological and anatomical characters is provided to distinguish genera and species.

Retamales, H. and T. Scharaschkin. 2015. Comparative leaf anatomy and micromorphology of the Chilean Myrtaceae: Taxonomic and ecological implications. *Flora* 217: 138-154.

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Foliar micromorphology and anatomy of *Ugni molinae* Turcz. (Myrtaceae), with particular reference to schizogenous secretory cavities

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Abstract

Background: *Ugni molinae* Turcz. is one of the most studied species of South American Myrtaceae due to its edible fruits and foliar medicinal compounds. However, there is no anatomical study of the leaves or secretory cavities. This paper seeks to describe the leaf micromorphology and anatomy of the species using standard protocols for light and scanning electron microscopy. Secretory cavities were anatomically characterized in young and mature leaves. Histochemical staining of the cavities was performed.

Results: The leaves of *U. molinae* are hypostomatic, have a wavy surface and possess scattered hairs. Leaf anatomical features include dorsiventral mesophyll, two to three layers of palisade parenchyma with abundant chloroplasts, calcium oxalate crystals and internal phloem in vascular bundles. Schizogenous secretory cavities are present on the abaxial surface and are mainly located on the margins of the leaves. Histochemical tests of these cavities suggest the presence of lipophilic substances.

Conclusions: This is the first study of secretory cavities in Chilean Myrtaceae. In general, micromorphological and anatomical characters are similar to other species of the family. The present findings could provide valuable anatomical information for future research in South American Myrtaceae.

Keywords: Anatomy; Myrtaceae; Secretory cavities; SEM; Terpenoids

Retamales, H. Scherson, R. and T. Scharaschkin. 2014. Foliar anatomy and micromorphology of *Ugni molinae* Turcz. (Myrtaceae) with particular reference to schizogenous secretory cavities. *Revista Chilena de Historia Natural* 87: 27.

MICROMORPHOLOGY AND ANATOMY OF LEAVES OF *SYZYGIUM FLORIBUNDUM* (MYRTACEAE: SYZYGIEAE), A RAINFOREST TREE ENDEMIC TO EASTERN AUSTRALIA

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Although species of *Syzygium* are abundant components of the rainforests in Queensland and New South Wales, little is known about the anatomy of the Australian taxa. Here we describe the foliar anatomy and micromorphology of *Syzygium floribundum* (syn: *Waterhousea floribunda*) using standard protocols for scanning electron microscopy (SEM) and light microscopy. *Syzygium floribundum* possesses dorsiventral leaves with cyclo-staurocytic stomata, single epidermis, internal phloem, rhombus-shaped calcium oxalate crystals and complex-open midrib. In general, leaf anatomical and micromorphological characters are common with some species of the tribe Syzygieae. However, this particular combination of leaf characters has not been reported in a species of the genus. The anatomy of the species is typical of mesophytic taxa.

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Retamales, H., Scherson, R. and T. Scharaschkin. 2014. Micromorphology and anatomy of leaves of *Syzygium floribundum* (Myrtaceae: Syzygieae), a rainforest tree endemic to eastern Australia. *Proceedings of the Royal Society of Queensland* 119: 45-52.

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Leaf micromorphology and anatomy of *Myrceugenia rufa* (Myrtaceae). An endemic coastal shrub of north-central Chile

Micromorfología y anatomía foliar de *Myrceugenia rufa* (Myrtaceae). Un arbusto costero endémico de la zona centro-norte de Chile

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ABSTRACT

Species of fleshy-fruited Myrtaceae are generally associated with humid environments and their vegetative anatomy is mainly mesophytic. *Myrceugenia rufa* is an endemic and rare species from arid zones of the coast of central Chile and there are no anatomical studies regarding its leaf anatomy and environmental adaptations. Here we describe the leaf micromorphology and anatomy of the species using standard protocols for light and scanning electron microscopy. The leaf anatomy of *M. rufa* matches that of other Myrtaceae, such as presence of druses, schizogenous secretory ducts and internal phloem. Leaves of *M. rufa* exhibit a double epidermis, thick cuticle, abundant unicellular hairs, large substomatal chambers covered by trichomes and a dense palisade parenchyma. Leaf characters of *M. rufa* confirm an anatomical adaptation to xerophytic environments.

KEYWORDS: Hairs, leaf anatomy, *Myrceugenia rufa*, Myrtaceae, SEM, xerophytic characters.

Retamales, H., Cabello. A., Serra, M.T. and T. Scharaschkin. 2014. Leaf anatomy and micromorphology of *Myrceugenia rufa* (Myrtaceae), an endemic shrub of the coast of Chile. *Gayana Botanica* 72(1): 76-83.

Boletín del Museo Nacional de Historia Natural, Chile, 63: 89-100 (2014)

ANATOMICAL STUDIES OF THE FLOWER, FRUIT AND SEEDS OF *MYRCEUGENIA RUFA* (MYRTACEAE)

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ABSTRACT

Myceugenia rufa is a rare and endemic species from the coast of central Chile. There are no published studies describing flower, fruit or seed anatomy. Forty-two accessions were collected from across the geographic range of the species. Reproductive structures were fixed, dehydrated, embedded in paraffin, sectioned and stained with Safranin O and Fast green. Anatomy of floral buds, mature flowers, fruits and seeds was described. Reproductive anatomy matches that of other Myrtaceae, such as presence of druses, internal phloem and schizogenous secretory cavities in buds, flowers, fruits and seeds. The anatomy and development of reproductive structures of *M. rufa* might enhance the understanding for future studies regarding natural reproduction and conservation programs.

Keywords: *Myrceugenia rufa*, reproductive anatomy, flower, fruit, seeds

Retamales, H., Cabello. A., Serra, M.T. and T. Scharaschkin. 2014. Anatomical studies of the flower, fruit and seeds of *Myrceugenia rufa* (Myrtaceae). *Boletin del Museo Nacional de Historia Natural* 63: 89-100.

Appendix 6. Abstracts of conference presentations during this PhD (*presenter)

Title: A comparative study of leaf anatomy and micromorphology in some Australasian and Chilean Myrtaceae (2013). Retamales, H. & T. Scharaschkin*

Format: Poster presentation

Conference: 'Systematics Without Borders', December 2013, Sydney, NSW, Australia

Myrtaceae is a pantropical angiosperm family, which is taxonomically diverse and ecologically significant in South America and Australasia. The taxa occur in a wide range of habitats and exhibit a notable morphological diversity. However, anatomical and micromorphological descriptions remain unknown in most of the species. This study describes and compares the leaf anatomy and micromorphology of some Chilean and Australasian species of Myrtaceae. The Chilean taxa include *Luma apiculata, Ugni molinae, Myrceugenia parvifolia, Myrceugenia rufa* and *Myrteola nummularia* and the Australasian taxa include *Acmena smithii, Decaspermum humile, Eugenia reinwardtiana, Syzygium australe, Syzygium forte, Austromyrtus floribunda* and *Waterhousea floribunda*. Leaf material was collected in the field and fixed in FAA. Processed material was embedded in paraffin, sectioned at 5µm using a Leica Rotary Microtome and stained with ruthenium red and toluidine blue following to Ruzin (1999). SEM photomicrographs were taken using a Quanta 200 SEM microscope for examination of leaf micromorphology. Notable features observed include cuticular ornamentation, stomatal type, druses and prismatic crystals, secretory cavities, number of layers of palisade parenchyma, internal phloem, double epidermis, bundle sheath extension, helical wall thickenings and scalariform perforation plates on vessels, type and distribution of hairs, epidermal cells shape and type of foliar colleters. In general, the anatomy and micromorphology of the species indicates features typical of mesophytic taxa. An exception is *Myrceugenia rufa*, whose morpho-anatomical features are typical of taxa occurring in xerophytic habitats. This is the first report on the leaf anatomy and micromorphology of most of these species. The anatomy and micromorphology of leaves in these species have the potential to be phylogenetically informative for the group.

Title: Character evolution in *Myrceugenia* (Myrtaceae): preliminary results using anatomical characters (2014). Retamales, H* & T. Scharaschkin.

Format: Oral presentation

Conference: 'Next Generation Systematics', November 2014, Palmerston North, New Zealand.

Myrceugenia Berg. is a South American genus of fleshy fruited Myrtaceae with ca. 40 species. Fourteen species occur in Chile and adjacent Argentina and 25 in eastern South America, mainly Brazil. Previous phylogenetic reconstructions based on molecular data alone have shown that these two disjunctive distributions are reciprocally monophyletic. The genus is considered monophyletic, except for *M. fernandeziana*. There is considerable variation in the anatomy within this genus and it is not known if the characters associated with xerophytic habit are due to convergent evolution or not. Here we rely on previous phylogenetic reconstructions in order to reconstruct the evolution of a number of anatomical characters. Some anatomical characters used in this study include the presence of trichomes, shape of crystals, shape of the midrib, number of epidermis layers, type of epidermal anticlinal walls, location of secretory cavities, presence of papillae, presence of sunken stomata, relative cuticle thickness and relative palisade/spongy parenchyma ratio. Evolution of xerophytic characters in *M. rufa*, *M. correifolia* and *M. euosma* are studied in detail. Some anatomical characters showed convergent evolution, while others are potential synapomorphies for the group. Results can provide meaningful background for further phylogenetic and taxonomic works in *Myrceugenia* by elucidating unambiguous synapomorphies for the clades within the genus.

Title: Phylogenetic relationships and character evolution in Australian Myrteae (Myrtaceae) (2015). Retamales, H* & T. Scharaschkin.

Format: Oral presentation

Conference: 'Botany 2015', July 2015, Edmonton, Alberta, Canada.

The tribe Myrteae (Myrtaceae) is widely distributed in Australasia and South America. In Australia, Myrteae is represented by ca. 100 species in 11 genera. Most species occur in rainforests and mesic habitats, but some species can be found in coastal sands and shrublands. To date, phylogenetic analyses in Myrteae have primarily focused on neotropical taxa. There is a lack of information about the morphology and anatomy of a number of genera endemic to Australia (*Austromyrtus*, *Lenwebbia*, *Lithomyrtus, Pilidiostigma*) and their systematic position is uncertain. A phylogenetic study of the tribe Myrteae, including representatives from Australia, South America, Central America, New Caledonia, New Zealand and southeast Asia was conducted using separated and combined analyses of DNA sequences and morpho-anatomical characters. Analysis of character history was undertaken in the software Mesquite 2.7 with maximum parsimony as optimization criterion. Preliminary results indicate that most Australian Myrteae (*Austromyrtus*, *Lenwebbia*, *Pilidiostigma*) have a typical mesophytic leaf, except for *Lithomyrtus*, which shows a number of xerophytic characters (e.g., a dense layer of hairs, thick epidermis, sunken stomata). Generally, character mapping suggested that the presence of xerophytic characters in lineages that are not closely related is mainly product of convergent evolution. This study will provide an updated phylogenetic framework for further investigations into Australian and South American species of fleshy-fruited Myrtaceae.

Title: Phylogeny and historical biogeography of Myrteae (Myrtaceae), with special reference to Chilean species (2016). Retamales, H., Scherson, R. & T. Scharaschkin*

Format: Oral presentation

Conference: 'Southern Connections', January 2016, Punta Arenas, Chile.

The predominantly southern hemisphere angiosperm family, Myrtaceae, is represented by 26 species in 10 genera in Chile, all of which (other than *Tepualia*) are in the tribe Myrteae. Chilean Myrteae occur in a diverse range of habitats. A number of species are endemic to Chile, a few also occur in Argentina but only one species is distributed widely to the east of the Andes. The monophyly, phylogenetic placement and historical biogeography of the Chilean Myrteae were investigated in this study. Phylogenetic analyses of DNA sequences from three loci for 90 species were performed using parsimony, maximum likelihood and Bayesian inference. Our results indicate that some Chilean genera are not monophyletic, e.g., *Myrceugenia fernandeziana* is sister to *Blepharocalyx cruckshanksii* and not associated with other *Myrceugenia*; *Myrcianthes coquimbensis* is associated with *Eugenia.* The position of *Tepualia* is confirmed in the tribe Metrosidereae. Our results largely agree with previous reconstructions of biogeographic scenarios, indicating multiple independent dispersal events of Chilean Myrteae to eastern Amazonian rainforests. Preliminary results also indicate trans-Tasman association of Australian-New Zealand Myrteae. The inclusion of additional genera restricted to Brazil, Argentina and Australasia and estimates of divergence age using appropriate fossil calibration is required to gain a more complete understanding of the historical biogeography of Myrteae.